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Transcriptional repression in quiescent cells is critical for the maintenance of cellular growth control. Previous work has shown the importance of E2F/Rb and E2F/p130 complexes in mediating repression of genes that are involved in cell cycle progression, however, the mechanisms of action of these complexes remains unclear.

The purpose of the work supported by this grant is to identify the mechanisms of p130 mediated transcriptional repression. To this end we have established that p130 recruits histone deacetylase as one mechanism of repression. Through a yeast two-hybrid screen, we have also found that the CtIP protein can interact with p130 and mediate a histone deacetylase independent mechanism of transcriptional repression. This mechanism involves the recruitment of the CtBP protein, which we have found to be capable of inhibiting the CBP transcriptional coactivator. These findings are significant not only to cancers related to deregulated E2F activity but also to breast cancers involving mutations of BRCA1 (Breast Cancer Susceptibility Gene 1)as the BRCA1 protein has been shown to interact functionally with CtIP.

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INTRODUCTION

E2F is a transcription factor that plays a critical role in directly inducing the expression of genes that are necessary for a cell cycle progression into S phase. During cellular quiescence, a time when cells are resting, the Retinoblastoma protein, Rb, or family member p130 is bound to E2F, forming an active complex that promotes the repression of E2F target genes. Mutations of Rb or the cyclin dependent kinase inhibitor p16, which regulates the activity of Rb, are common abnormalities in human breast cancer indicating that the E2F pathway may be involved in the generation of breast cancer. Thus further elucidation of the E2F/Rb and E2F/p130 complexes and how they act to control cell growth may benefit the diagnosis and treatment of breast cancer. Our research therefore aims to elucidate the mechanisms by which Rb and family member, p130, convert E2F from a positive acting factor into a negative acting transcriptional repression complex. Importantly, our findings are not only relevant to the Rb/p130 transcriptional repressor proteins but also to the breast cancer susceptibility protein, BRCA1, which has been proposed to play a role in transcriptional repression.

BODY:

Objective 1: To identify the mechanism of p130 mediated repression.

The goal of the first objective was to identify potential mechanisms of p130 mediated repression. Given that histone deacetylase (HDAC) was known to mediate repression of the MAD transcriptional repressor (Laherty et al. 1997), we hypothesized that p130 might interact with HDAC or with other proteins that form larger complexes with HDAC to mediate repression. Indeed, we found that HDAC can interact with wild type p130. Interestingly, the p130 C894F mutant that also interacts with HDAC, is inhibited in its ability to repress transcription suggesting that simple interaction with HDAC may not be sufficient to mediate repression (Figure 1.1).

Further evidence that p130 can repress transcription through a recruitment of HDAC arises from the finding that p130 and HDAC have a synergistic effect on transcriptional repression (Figure 1.2). Moreover, the addition of trichostatin A (TSA), a potent HDAC inhibitor, to starved REF52 (Rat Embryonic Fibroblasts) cells is capable of abolishing endogenous p130-mediated repression of the Cyclin E promoter but not the E2F1 promoter (Figure 1.3). The fact that one promoter, Cyclin E, was derepressed by the addition of the HDAC inhibitor indicates that p130 uses HDAC-dependent mechanisms to repress some promoters. However, other promoters such as E2F1 are clearly repressed by other mechanisms.

Given that p130 seemed capable of repressing certain promoters through HDAC-independent mechanisms, we performed a yeast two-hybrid assay to find proteins that were capable of interacting with p130 and capable of mediating transcriptional repression. We identified several proteins in this screen such as cyclin D1, cyclin D3, E2F4, and E2F5, proteins already known to interact with p130. Other interesting proteins were Tumor Necrosis Factor type-1 receptor associated protein, a homeobox transcription factor similar to the LBX-1 transcription factor, a protein called DIP (DP3-interacting protein), Rad50-interacting protein, and a protein called CtIP (CtBP-interacting protein). We chose to examine the CtIP protein further and have found that CtIP can itself repress transcription and that this repression is due at least in part to its ability to recruit the CtBP

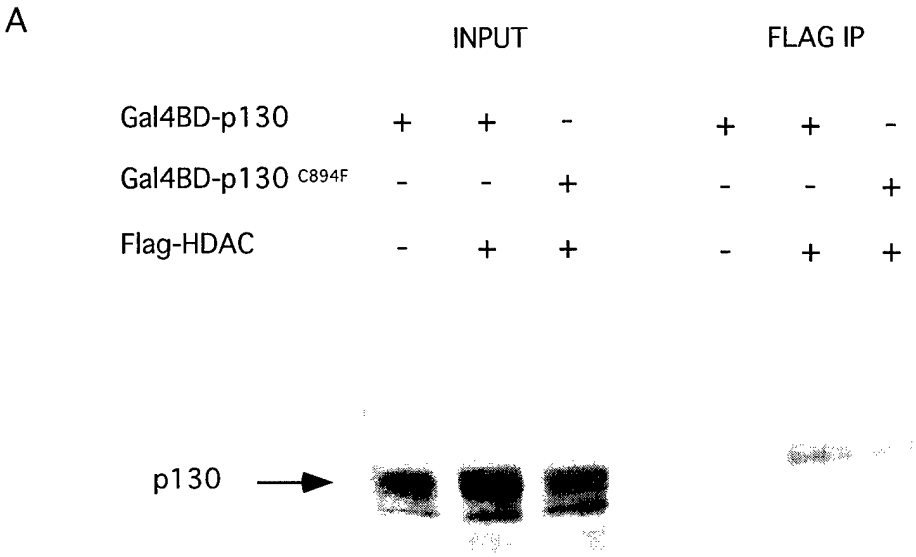


Figure 1.1 p130 interacts with histone deacetylase. A) C33A cells were transfected with Flag-HDAC and Gal4BD-p130 or the pocket mutant p130 ^{C894F}. Extracts were immunoprecipitated (IP) with Flag antibody and western blotted using Gal4BD antibody. Input lanes represent 10% of the material used in the IP.

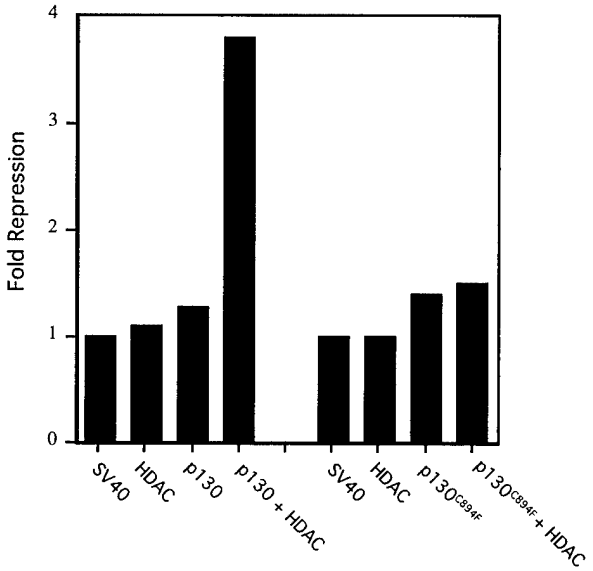


Figure 1.2 Histone deacetylase and p130 act synergistically to repress transcription. C33A cells were transfected with 0.5 μg SV40 and 200 ng of p130, 1 μg HDAC, or the two together. The same transfections were performed using p130 ^{C894F}. Total DNA transfected was kept constant with the pCDNA3 control vector. CAT assays were performed and adjusted for transfection efficiency. Data is graphed as a fold repression. Representative experiments are shown.

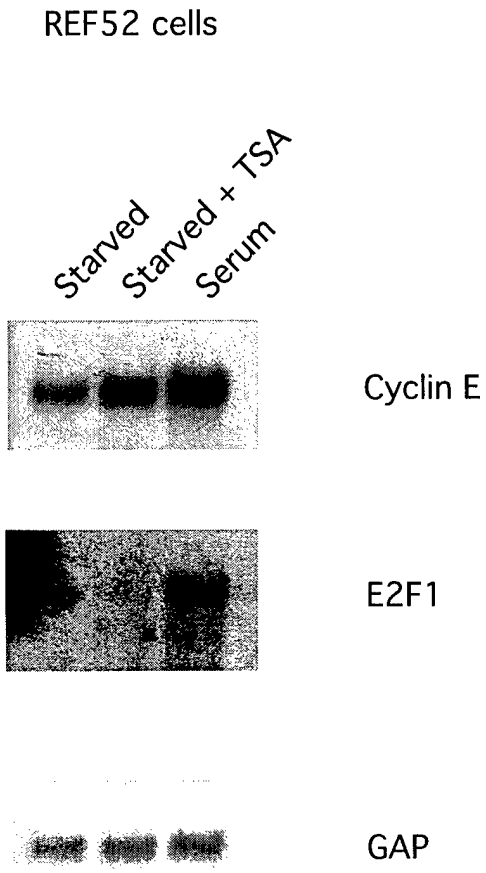


Figure 1.3 Trichostatin A derepresses some E2F target genes. REF52 cells were serum starved for 48 hours followed by TSA(100nM) treatment for 24 hours or serum stimulation for 20 hours. Poly A RNA was harvested and northern blot performed using probes for Cyclin E, E2F1, and GAP as a loading control. Cyclin E blot was exposed for 16 hours. E2F1 blot was exposed for 7 days.

(C-terminal binding protein) transcriptional corepressor protein. This finding revealed that p130 can mediate repression both through histone deacetylase dependent mechanisms and also through the recruitment of the CtIP/CtBP corepressor complex (for more details on these findings please refer to the appended paper (Meloni et al. 1999).)

Objective 2: To identify a region of p130 responsible for p130 mediated repression.

The original goal of this objective was to separate the ability of p130 to bind to E2F from its ability to repress transcription. This objective aimed to separate the two different functions of p130, the ability to inhibit the positive function of E2F and the ability to actively repress transcription. Since the original description of this objective, several papers have been published revealing that the two functions of Rb are distinguishable (Chen and Wang 2000; Dahiya et al. 2000; Dick et al. 2000; Ross et al. 2001). We had originally proposed a yeast reverse two-hybrid assay as an assay for identifying p130 mutants that were capable of binding to E2F but not to proteins that contained the typical Rb binding motif, the LXCXE amino acid motif. Using the crystal structure of Rb, three separate groups created mutants of Rb that seemed likely to abolish the LXCXE-binding pocket domain of Rb. The results of these papers identified that mutants of Rb that could not bind LXCXE-motif proteins were defective for active transcriptional repression but retained the ability to inhibit the positive activation function of E2F. In light of these findings, we have not pursued this line of investigation.

Revised Objective 2: To examine the role of CtIP in mediating transcriptional repression.

The finding that p130 can mediate repression through a recruitment of CtIP was made even more interesting in light of the finding that the BRCA1 (Breast Cancer Susceptibility) protein could also interact with CtIP. CtIP interacts with the BRCT (BRCA1 C-terminal) repeats of BRCA1. Interestingly, mutation of these BRCT repeats can be found in human tumors and these mutations abolish the interaction of BRCA1 with CtIP (Yu et al. 1998; Li et al. 1999). It has been further shown that CtIP/CtBP

complexes are involved in the BRCA1-mediated repression of the DNA damage response gene, GADD45. Moreover, the ATM (ataxia telangiectasia) kinase can phosphorylate CtIP triggering the dissociation of CtIP and BRCA1 and derepressing the GADD45 gene. (Li et al. 2000). The importance of the CtIP protein in both p130 as well as BRCA1-mediated transcriptional repression prompted the further examination of a mechanism for CtIP-mediated transcriptional repression.

We found that, in part, CtIP represses transcription through a recruitment of the CtBP protein (Meloni et al. 1999). We further attempted to find other components of this CtIP repressor complex via a yeast two-hybrid screen however the full length CtIP protein seems to be toxic as a Gal4 DNA binding domain fusion protein and few transformants were obtained. In addition, the CtIP-Gal4 DNA binding domain fusion protein activates transcription, yielding high background in the screen. As a result, no further proteins that interact with CtIP could be identified in this manner. To date CtIP has been shown to interact with only a small handful of proteins, Rb (retinoblastoma), p130, BRCA1, and CtBP. Future work aims to identify other members of the CtIP complex by passing cellular extracts over a matrix column containing bound CtIP protein made by baculovirus expression. Proteins that stick to the column would then be purified and identified by microsequencing.

We next wanted to examine the expression of CtIP in different breast cancer cell lines. Northern analysis of CtIP message indicates that CtIP RNA message may be differentially expressed in tumor cell lines (Fusco et al. 1998). To examine CtIP in tumor cell lines at the protein level we made a rabbit antibody against CtIP. One rabbit made an antibody that is specific to CtIP and that antibody is in the process of being purified. Once purified, this antibody can be used to analyze different breast cancer tissues or cell lines for the expression of the CtIP protein.

Objective 3: To investigate the possibility that E2F4 is mutated in human breast carcinomas.

Several breast cancer cell lines were obtained from ATCC. Many of these lines were analyzed for the expression of E2F4, however none tested were found to lack

expression or express mutant forms of the protein. As the MD fellow that guided this objective, Dr. Paul Kelly Marcom, has left the lab to take a position in the Oncology department at Duke University Medical Center, no further examination of this objective has been undertaken.

Revised Objective 3: To examine the function of the CtIP/CtBP complex in transcriptional repression.

Given the importance of the CtIP/CtBP complex in transcriptional repression mediated by both p130 as well as BRCA1, we next wanted to examine the mechanism of action of this complex. Since CtIP seemed to be acting as a repressor of transcription partly due to its ability to recruit CtBP, we further examined the function of CtBP. Using mutants of CtBP, we found that the ability of CtBP to repress transcription requires a functional N-terminal (amino-terminal) domain. This region of CtBP is known to be important for interaction with a class of proteins that contain a PLDLS amino acid motif. Given that transcriptional repression through CtBP requires the domain that is involved in interaction with PLDLS-containing proteins, we reasoned that CtBP might repress transcription by recruiting a PLDLS motif containing protein. We have found that the CBP protein, a histone acetyltransferase coactivator, contains a PLDLS motif and can interact with CtBP. The result of this interaction is the inhibition of the histone acetyltransferase activity of the CBP protein. The finding that CtBP can inhibit the acetyltransferase activity of CBP is important because an increasing body of evidence suggests that many transcriptional corepressors recruit both histone deacetylase and CtBP to repress transcription. Our finding that CtBP can inhibit histone acetylase activity implies that transcriptional repression involves both the recruitment of histone deacetylase as well as inhibition of the opposing enzyme, histone acetylase, which is found at virtually all promoters. This dual action might allow a more complete transcriptional repression, preventing further acetylation of histones or other targets after the action of the histone deacetylase. A more detailed description of the findings covered by this objective can be found in the appended manuscript (Meloni PNAS submitted).

Key Research Accomplishments:

1) Rb and p130 can immunoprecipitate histone deacetylase indicating that the proteins interact.

2) The histone deacetylase inhibitor, TSA, is incapable of derepressing most E2F target genes thereby establishing the likelihood of a histone deacetylase independent mechanism of transcriptional repression for Rb/p130.

3) We identified a protein, CtIP, that interacts ith p130 in a yeast to-hybrid screen and by co-immunoprecipitation.

4) We characterized CtIP as having the activity of a transcriptional co-repressor.

5) CtIP can repress transcription, in part through recruitment of the CtBP protein.

6) We have established that CtBP has a histone deacetylase independent mechanism of transcriptional repression.

7) We found that the tumor virus protein, E1A has the ability to prevent CtBP from repressing transcription.

8) We defined a domain on CtBP (the PID domain) that is important for transcriptional repression and for interaction with a class of proteins that contain a PLDLS amino-acid motif.

9) CtBP can interact with the CBP transcriptional co-activator.

10) CtBP can inhibit the histone acetylase activity of the CBP protein independently of any possible dehydrogenase activity that CtBP may have.

List of Reportable Outcomes

Manuscripts

1) Meloni A.R., Smith E.J., and Nevins J.R. A mechanism for Rb/p130-mediated transcription repression involving recruitment of the CtBP corepressor" PNAS 96, 9574-9579, August 1999.

2) Meloni A.R., Hamilton M.R., Lai C.H., Yao T.P., and Nevins J.R. The CtBP Co-Repressor Inhibits Histone Acetylase activity of CBP. Resubmitted to PNAS January, 2001.

3) Kegel K.B., Meloni A.R., Yun Y.Y., Kim J., Doyle E., Sapp E., Want Y., Qin Z.H., Chen D., Aronin N., and DiFiglia M. Huntington is present in nuclear bodies and nuclear matrix and interacts with the transcriptional corepressor C-terminal binding protein. Submitted to JBC April 2001.

Posters

1) Meloni A.R., and Nevins J.R. A Mchanism for Rb/p130 Mediated Transcription Repression. Duke University Graduate Student Symposium. 1995-2000.

2) Meloni A.R., and Nevins J.R. A Mchanism for Rb/p130 Mediated Transcription Repression. ISREC Conference: Cancer and the Cell Cycle. Lausanne, Sitzerland. January 1999.

3) Meloni A.R., and Nevins J.R. A Mchanism for Rb/p130 Mediated Transcription Repression Involving Recruitment of the CtIP/CtBP Co-repressor Complex. Keystone Sympoaia: Cancer, Cell Cycle, and Therapeutics. Steamboat, CO. January 2000.

4) Meloni A.R., Hamilton M.R., Lai C.H., Yao T.P., and Nevins J.R. CtBP Represses Transcription by Inhibiting the Histone Acetylase activity of CBP. Era of Hope Symposia. Atlanta, GA. June 2000.

Other

1) Received a Ph.D. from Duke University in May 2001.

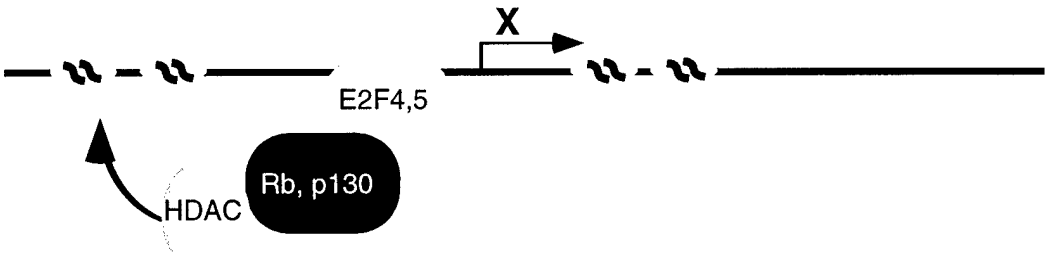
2) A Postdoctoral research position in the lab of Dr. Albert Baldwin was applied for based on the research supported by this grant.

Conclusions

As described by the model on the following page, we propose that on some promoters, E2F/Rb or E2F/p130 complexes can recruit histone deacetylase to modify chromatin structure at target promoters and prevent accessibility of transcription machinery resulting in repression [Brehm, 1998 #2468; Ferreira, 1998 #2493; Luo, 1998 #2534; Magnaghi-Jaulin, 1998 #2535]. However, under other conditions, E2F/Rb or E2F/p130 complexes may bind to CtIP through a pocket domain/LXCXE motif interaction [Meloni, 1999 #2781]. CtIP would then interact with the PID domain of one molecule of the CtBP dimer, through a PLDLS motif, while the PID domain of the other molecule of CtBP remained free to associate with and inactivate the transcriptional co-activator protein, CBP that exists on most promoters. The inactivation of CBP histone acetylase activity by CtBP therefore would prevent transcriptional activation by inhibiting acetylation of histones, pushing the overall state of the nucleosomes towards deacetylation, favoring transcription repression. This model represents a potential new mechanism for transcription repression. As nearly all promoters use CBP to coactivate transcription, inactivation of these coactivators could result in promoter silencing. It remains to be determined however if this mechanism alone is sufficient to repress transcription or if it contributes to a more complete repression together with other mechanisms that might recruit histone deacetylase.

In addition to the elucidation of a mechanism for Rb family-mediated repression, this mechanism is also likely to be important in transcriptional repression mediated through the breast cancer susceptibility gene, BRCA1, which also recruits the CtIP/CtBP complex. It is therefore possible that mutations in either CtIP or CtBP may contribute to the generation of breast cancer and may ultimately be useful as prognostic indicators or targets for therapy.

Recruitment of Histone Deacetylase



Recruitment of a Histone Acetylase Inhibitory Complex

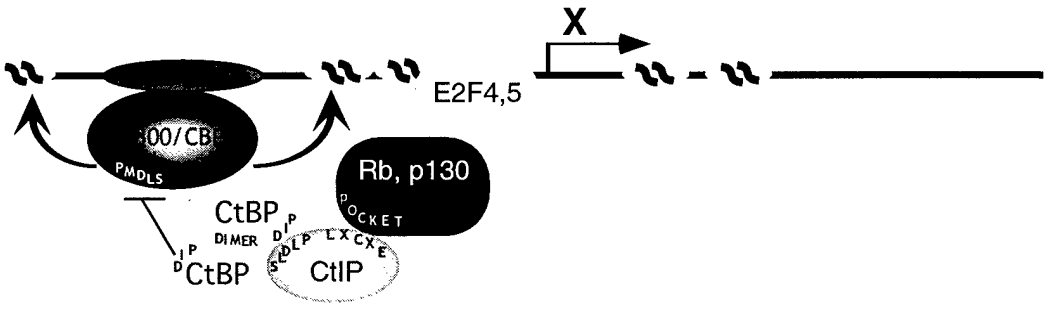


Figure 2.1 Complementary mechanisms for E2F/Rb mediated transcriptional repression. E2F target promoters can be repressed in two fashions. Histone deacetylase is recruited to promoters that contain E2F/Rb or E2F/p130 complexes through an interaction with Rb or p130. Histone deacetylase then modifies the histones proximal to the promoter causing transcriptional silencing. Rb and p130 recruit CtIP/CtBP to E2F complexes. CtIP bridges the interaction between CtBP and the E2F/Rb complex. CtBP, acting as a dimer, then functions by inhibiting p300/CBP bound to the promoter through basal transcription machinery or other transcription factors.

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the transcription regulation of p21 is disrupted upon DNA damage. *J Biol Chem* **274**: 11334-11338.

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APPENDICES

A mechanism for Rb/p130-mediated transcription repression involving recruitment of the CtBP corepressor.

The CtBP Co-repressor Inhibits Histone Acetylase Activity of CBP.

Prot. Natl. Acad. Sci. USA
Vol. 96, pp. 9574–9579, August 1999
Biochemistry

A mechanism for Rb/p130-mediated transcription repression involving recruitment of the CtBP corepressor

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Edited by Ed Harlow, Harvard Medical School, Charlestown, MA, and approved June 28, 1999 (received for review April 26, 1999)

ABSTRACT **Previous work has demonstrated the critical role for transcription repression in quiescent cells through the action of E2F-Rb or E2F-p130 complexes. Recent studies have shown that at least one mechanism for this repression involves the recruitment of histone deacetylase. Nevertheless, these studies also suggest that other events likely contribute to E2F/Rb-mediated repression. Using a yeast two-hybrid screen to identify proteins that specifically interact with the Rb-related p130 protein, we demonstrate that p130, as well as Rb, interacts with a protein known as CtIP. This interaction depends on the p130 pocket domain, which is important for repression activity, as well as an LXCXE sequence within CtIP, a motif previously shown to mediate interactions of viral proteins with Rb. CtIP interacts with CtBP, a protein named for its ability to interact with the C-terminal sequences of adenovirus E1A. Recent work has demonstrated that the *Drosophila* homologue of CtBP is a transcriptional corepressor for Hairy, Knirps, and Snail. We now show that both CtIP and CtBP can efficiently repress transcription when recruited to a promoter by the Gal4 DNA binding domain, thereby identifying them as corepressor proteins. Moreover, the full repression activity of CtIP requires a PLDLS domain that is also necessary for the interaction with CtBP. We propose that E2F-mediated repression involves at least two events, either the recruitment of a histone deacetylase or the recruitment of the CtIP/CtBP corepressor complex.**

The control of the early events of cell proliferation through the action of the G1 cyclin-dependent kinases, leading to the phosphorylation of Rb and related proteins, and the subsequent accumulation of E2F transcription factor activity is now well established (for reviews, see refs. 1–6). It is also evident that most, if not all, human cancers arise as a result of the disruption of this pathway, either through the activation of positive acting components such as the G1 cyclins or the inactivation of negative-acting components such as p53, Rb, and the cyclin kinase inhibitors (6, 7).

E2F transcription activity is now recognized to be a complex array of DNA binding activities that function both as transcriptional activating proteins as well as transcription repressors (8, 9). The E2F4 and E2F5 proteins, which specifically associate with the Rb-related p130 protein in quiescent cells (10), function to repress transcription of various genes encoding proteins important for cell growth. In contrast, the E2F1, E2F2, and E2F3 proteins are tightly regulated by cell proliferation, accumulate as cells progress through mid- to late G1, and appear to function as positive regulators of transcription. The complexity of E2F transcription control is illustrated by the fact that the E2F1, E2F2, and E2F3 genes are repressed in quiescent cells through the action of E2F4 or E2F5 complexes containing Rb or p130. In addition to the E2F1, E2F2, and E2F3 genes, the targets for E2F-mediated repression include a very large number of genes that encode proteins that guide cell cycle progression and that participate directly in DNA replication (8, 9).

Initial studies of Dean and colleagues clearly demonstrated that the role of Rb in controlling E2F-dependent transcription was not merely an inhibition of positive activation of transcription but, rather, that E2F/Rb-mediated repression was a dominant event, capable of shutting off an otherwise active promoter (11, 12). Indeed, a series of recent reports has provided evidence that one mechanism for this Rb-mediated repression involves an ability of Rb to recruit histone deacetylase to E2F-site containing promoters, presumably resulting in an alteration of chromatin conformation that hinders transcription (13–15).

Nevertheless, despite the evidence implicating histone deacetylase recruitment as a mechanism for Rb-mediated repression, several observations suggest that additional events may contribute to the repression. For example, many genes subject to E2F/Rb-mediated repression are not derepressed by treatment with the histone deacetylase inhibitor trichostatin A (13). Moreover, although the recruitment of histone deacetylase is effective in repressing some promoters, others appear to be unaffected. Based on these observations, it would appear that a histone deacetylase-independent mechanism of transcriptional repression contributes to the Rb control of transcription.

To further explore the mechanistic basis for Rb-mediated repression, we have used a yeast two-hybrid screen to identify proteins that specifically interact with the p130 protein. In so doing, we have identified a protein known as CtIP that interacts with p130 dependent on the p130 pocket domain. CtIP has previously been described (16) as a protein that interacts with CtBP, an adenovirus E1A-interacting protein (17), and CtBP has recently been shown to function as a corepressor in *Drosophila*. We show here that the CtIP protein can itself repress transcription and that this repression is caused, at least in part, by its ability to recruit the CtBP corepressor. Rb/p130-mediated repression therefore functions not only through histone deacetylase activity but also through a CtIP/CtBP repressor complex.

MATERIALS AND METHODS

Cell Culture. C33A cells were grown in DMEM containing 10% fetal bovine serum.

Plasmids and Reagents. The SV40 promoter containing upstream Gal4 sites (pSVECG) was a kind gift from D. Dean (Washington University, St. Louis) (12). The MLP with Gal4 sites was a kind gift from D. Dean and R. Eisenman (Fred Hutchinson Cancer Research Center, Seattle), the Gal4-

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Abbreviations: β-gal, β-galactosidase; CAT, chloramphenicol acetyltransferase.

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HDAC expression plasmid was a kind gift from D. Reinberg (Robert Wood Johnson Medical School, Piscataway, NJ) (18), and the RbΔp34 plasmid was a kind gift from R. Bremner (University of Toronto) (19). The Gal4Rb plasmids were created in several steps. The *Pvu*II Fragment of Rb was first subcloned into the *Sma*I site of the pGBT9 vector. The *Bsa*H-*Bgl*II fragment of Rb then was cloned into the *Cla*I and *Bgl*II sites of the pSP70 cloning vector. An *Eco*RI fragment was isolated from Rb-pSP70 (including the upstream *Eco*RI site from the polylinker) and was cloned into the Rb-pGBT9 vector, creating a full length Gal4Rb-pGBT9 plasmid used for expression in yeast. A *Hin*dIII fragment was isolated from the Gal4Rb-pGBT9 plasmid and was cloned into the same site in pCDNA3 to create the Gal4Rb-pCDNA3 plasmid used for expression in mammalian cells. The Gal4p130 plasmid was created by digesting pBluescript SK(+) (Stratagene), which contained full length p130 in the *Hin*dIII site, with *Bam*HI and *Sal*I. This fragment then was cloned into the *Bam*HI and *Sal*I sites of the pGBT9 vector. To put the fragment in frame with the Gal4 DNA binding domain, the p130-pGBT9 plasmid was cut with *Eag*I and *Sma*I and was religated, creating Gal4p130-pGBT9 that was used for expression in yeast. To create Gal4p130 for expression in mammalian cells, a *Hin*dIII fragment of Gal4p130-pGBT9, including the Gal4DBD, was cloned into the *Hin*dIII site of pCDNA3. The $p130^{C894F}$ and Rb^{C706F} mutants were made by using the CLONTECH Transformer Site-Directed Mutagenesis Kit and the primers 5′ CAA ATT ATG ATG TTT TCC ATG TAT GG 3′ for Rb and 5′ CAG TTA TTA ATG TTT GCC ATT TAT GTG 3′ for p130. The p130 pocket domain-containing plasmid was made by PCR using primers with *Bam*HI sites followed by 5′ CCA GTT TCT ACA GCT ACG CAT 3′ and 5′ TTA ATG TGG GGA AAT GTA GAC 3′. The *Bam*HI fragment was cloned into the *Bam*HI site of a pCDNA3-Gal4DBD plasmid. The pCDNA-3-Gal4DBD plasmid was made by cloning the *Hin*dIII-*Sal*I fragment from pGBT9 into the *Hin*dIII and *Xho*I sites of pCDNA3. The Gal4AD CtIP clone was isolated from the two-hybrid screen. Full length Gal4AD CtIP was created by PCR using the Gal4AD human fetal liver library as the template with the primers 5′ GTT ACT GTA ATA GAT ACA AA 3′ and 5′ AAA AGG GCC CCT ATG TCT TCT GCT CCT TGC 3′. The PCR product was cut with *Bsr*GI and *Apa*I and was subcloned into the same sites in Gal4AD CtIP. The resulting full length Gal4AD CtIP was sequenced to confirm that no mutations were introduced. Myc-CtIP was created by subcloning the *Bgl*II-*Apa*I fragment of Gal4AD CtIP into the *Bam*HI and *Apa*I sites of the pCDNA3-Myc vector. The Myc-CtIP ΔLXCXE was created by cutting the Myc-CtIP vector with *Bam*HI and *Hpa*I, treating with Klenow to fill the DNA ends, and then religating with DNA ligase. The Myc-CtIP ΔPLDLS vector was made in several steps. By using PCR with the primers 5′ TTT AGC AAC ACT TGT 3′ and 5′ AAA AGG ATC CTT TAT CCA TCA CAC 3′, an N-terminal fragment of CtIP was made. This fragment was subcloned into pBluescript SK(+) at the *Xba*I and *Bam*HI sites. A second fragment was created by PCR using the primers 5′ AAA AGG ATC CGA TCG ATT TTC AGC 3′ and 5′ AAA AGG GCC CCT ATG TCT TCT GCT CCT TGC 3′. This fragment was subcloned behind the first fragment in the pBluescript SK(+) *Bam*HI and *Apa*I sites. The whole fragment of CtIP, which now contained a deletion of the PLDLS motif, was cut out of pBluescript SK(+) by using *Xba*I and *Apa*I and was subcloned into the same sites of the Myc-CtIP vector. Gal4BD CtIP was made by subcloning the *Bam*HI-*Apa*I fragment from Myc-CtIP into the *Bgl*II and *Apa*I sites of the pCDNA3 Gal4DBD plasmid. Gal4BD CtIP ΔPLDLS was generated by subcloning the *Bam*HI-*Apa*I fragment from Myc-CtIP ΔPLDLS into the *Bgl*II and *Apa*I sites of the pCDNA3 Gal4DBD plasmid. Gal4BD CtBP was made by PCR using the primers 5′ AAA AGA ATT CAT GGG CAG CTC GCA CTT GCT 3′ and 5′ AAA ATC TAG ACT ACA ACT GGT CAC TGG CGT 3′ and using the CtBP clone that was a kind gift from G. Chinnadurai (Saint Louis University) as a template. The PCR product was digested with *Eco*RI and *Xba*I and was cloned into the same sites of the pCDNA3 Gal4DBD plasmid.

Repression Assays. C33A cells were transiently transfected by the calcium phosphate method with the pSVECG reporter and Gal4DBD fusion proteins. One microgram of the β-galactosidase (β-gal) plasmid was cotransfected as a control for transfection efficiency. After 15 hours, cells were washed twice with PBS and were allowed to recover in DMEM with 10% serum. Forty hours posttransfection, cells were harvested, and chloramphenicol acetyltransferase (CAT) assays were performed as described (20), although extracts were not heat inactivated. CAT assay reaction mixture included 75 μl of extract, 75 μl of 1M Tris·Cl (7.8) 1 μl of C^{14}-labeled chloramphenicol (1 mCi/ml), and 30 μl of acetyl CoA (3.5 mg/ml in H_2O). Reactions were incubated at 37° for 3.5 hours. CAT values were normalized relative to the vector alone control β-gal values. β-gal activity was measured by adding 10 μl of the extract prepared for the CAT assays to 590 μl of 0.1 mg/ml chlorophenol red-β-D-galactopyranoside in lac Z buffer (60 mM Na_2HPO_4/40 mM NaH_2PO_4/10 mM KCl/1 mM $MgSO_4$/38 mM 2-mercaptoethanol, pH 7.0). The absorbance of each sample was measured at 570 nm. In all instances of comparison, Western blot analyses were performed to determine that equal protein was expressed.

Yeast Two-Hybrid Screen. The yeast two-hybrid screen was performed as recommended in the CLONTECH protocol. Inserts from positive clones were sequenced according to Sequenase Kit (United States Biochemical) instructions.

Immunoprecipitations. C33A cells were transiently transfected by the calcium phosphate method. After 15 hours of transfection, cells were washed twice with DMEM, and then complete media was replaced. Forty hours posttransfection, cells were harvested and lysed in IP buffer containing 50 mM Tris·HCl (ph 7.4), 250 mM NaCl, 5 mM EDTA, 0.1% NP40, and the protease inhibitors Leupeptin at 1 μg/ml, Aprotinin at 1 μg/ml, at Pepstatin 1 μg/ml, and PMSF or Perfablock (Boehringer Mannheim) at 1 mM. Extracts were precleared by incubating with protein A agarose beads (Calbiochem) for 1 hour and then were centrifuged at 20,000 × *g* for 10 minutes. An aliquot of the sample (10%) was used as input, and 10% was used in a β-gal assay. The amount of extract used in the immunoprecipitation was normalized based on the β-gal values. One microgram of the appropriate antibody was added to precleared extracts and was allowed to mix at 4°C for 3 hours. Protein A agarose beads then were added and allowed to mix at 4°C for 1.5 hours. Samples then were washed 4 times at 4°C with 1 ml of IP buffer and were run on an SDS polyacrylamide gel.

RESULTS

Recent work has provided evidence for a mechanism for E2F/Rb-mediated repression that involves the recruitment of histone deacetylase (13–15). In particular, these studies demonstrated an ability of Rb to physically interact with HDAC that coincided with the ability of Rb to repress transcription. Nevertheless, this work also suggested that additional events may contribute to the ability of Rb to repress transcription. For instance, whereas the addition of the HDAC inhibitor trichostatin A reversed the repression of the adenovirus major late promoter, trichostatin A had little effect on the ability of Rb to repress transcription of the SV40 or TK promoter (13). Moreover, although the MAD protein, which is known to repress transcription through the recruitment of HDAC (21), could efficiently repress the major late promoter, it had no effect on the SV40 promoter (13). Although the distinction between these promoters remains unclear, the apparent in-

sensitivity of the SV40 promoter to the recruitment of HDAC provides an assay to examine mechanisms of Rb-mediated repression that are independent of HDAC recruitment.

To further explore the basis for HDAC-independent E2F/Rb-mediated repression, we assayed wild-type and mutant versions of the Rb family proteins for their ability to repress transcription driven by an SV40 promoter that also contained upstream Gal4 sites (Fig. 1*A*). As shown in Fig. 1*B*, fusion proteins linking either Rb or p130 to the Gal4 DNA binding domain were capable of repressing transcription of the reporter in C33A cells. Similarly, a mutant of Rb that deletes eight sites for phosphorylation by cdc2 (RbΔp34) is an even more efficient repressor of transcription than wild-type Rb (19). As previously published for Rb (12), the pocket domain of p130, when tethered to the Gal4 DNA binding domain, repressed transcription, indicating that the pocket domain is sufficient for repression of the SV40 promoter. An Rb mutation found in human tumors, involving a Cys to Phe change at position 706 within the pocket domain (Rb^{C706F}), has been shown to disrupt the structure of the pocket domain and therefore to abolish the interaction of Rb with the viral oncoproteins E1A and T antigen (22). As previously shown by Dean and colleagues (12), the Rb^{C706F} mutant failed to repress transcription of the SV40 promoter (Fig. 1*B*). Likewise, a p130 mutant constructed to contain the equivalent alteration in the homologous sequence ($p130^{C894F}$) also failed to repress transcription (Fig. 1*B*).

Rb and p130 Interact with CtIP. Given the indication from previous work that E2F/Rb-mediated repression could not be fully explained by recruitment of histone deacetylase, we initiated a search for other proteins that might be involved in an HDAC-independent transcriptional repression by Rb or p130. We used a full length p130 protein in a yeast two-hybrid assay to screen for potential protein partners of p130 that could mediate this HDAC-independent repression. The HF7C yeast strain was transformed with a plasmid encoding a Gal4 DNA binding domain-p130 fusion protein together with a human fetal liver cDNA library that incorporated the Gal4 activation domain. Forty positive transformants yielded 11 different clones encoding proteins that interacted with p130. Among the positive clones were cyclin D1, cyclin D3, E2F4, and E2F5, proteins known to specifically interact with Rb/p130.

In addition to these anticipated interacting proteins, one clone was found to encode the first 800 amino acids of a protein previously identified as CtIP. CtIP (C-terminal interacting protein) was originally recovered in a yeast two-hybrid screen as a partner of a protein known as CtBP (C-terminal binding protein) (16), a protein that binds to the C terminus of adenovirus E1A (23). The N-terminal portion of CtIP contains an LXCXE sequence (Fig. 2*A*), a motif found in the viral oncoproteins E1A, T antigen, and E7, as well as the D type cyclins, and that mediates the interaction with the Rb family proteins. Given the presence of the LXCXE motif in CtIP, we tested the ability of CtIP to interact with Rb in the two-hybrid assay. Fig. 2*B* shows that CtIP can specifically interact with p130 and Rb but not with an unrelated yeast protein, KSS1.

We also used a coimmunoprecipitation assay to measure the ability of CtIP to interact with p130, as well as to define the sequences important in each protein for the interaction. As shown in Fig. 2*C*, wild-type p130 could be recovered in an immunoprecipitate with the CtIP protein, but a pocket disrupting mutant p130 protein ($p130^{C894F}$) could not. In addition, p130 could be found to associate with the wild-type CtIP but not with a mutant of CtIP in which the first 170 amino acids, including the LXCXE domain, was deleted (Fig. 2*D*). It thus appears clear that CtIP interacts with p130, as well as Rb, and does so via an LXCXE-pocket domain interaction.

The observation that p130 interacts with CtIP, dependent on the pocket domain that is also required for p130-mediated repression, suggested a possible role for CtIP in transcriptional repression. To explore such a function, a fusion protein containing the Gal4 DNA binding domain linked to CtIP was created and assayed for its ability to repress the SV40 promoter reporter construct. As shown in Fig. 3, the Gal4-CtIP fusion protein was indeed active as a repressor; in fact, the Gal4-CtIP protein was as efficient as the Gal4-p130 fusion protein in the repression of the SV40 promoter. Based on all of these results, we conclude that recruitment of the CtIP protein represents an alternative mechanism, in addition to the recruitment of histone deacetylase, for p130-mediated repression.

CtIP Recruits the CtBP CoRepressor. CtIP was isolated based on its interaction with CtBP, a protein identified as an adenovirus E1A-binding protein (16). More recently, a *Drosophila* homologue of CtBP has been identified and shown to function as a corepressor for the transcriptional regulatory proteins Hairy, Knirps, and Snail (24–26). These observations thus suggested the possibility that CtIP might function in E2F/p130-mediated repression by recruiting the CtBP protein. To investigate this possibility, we first examined the ability of CtIP to interact with CtBP. C33A cells were cotransfected with a plasmid encoding a Myc-tagged CtIP protein and a plasmid encoding Gal4-CtBP. Cells then were assayed for an interaction of the two proteins by immunoprecipitating Gal4-CtBP and then assaying for the presence of CtIP in the immunoprecipitates by Western blotting. As shown in Fig. 4*A*, wild-type CtIP was indeed recovered in the CtBP immunoprecipitate.

Previous work has demonstrated that the interaction of CtBP with E1A, or the interaction of the *Drosophila* CtBP with Hairy, Knirps, and Snail, depends on a PLDLS sequence found within these interacting proteins. Examination of the CtIP

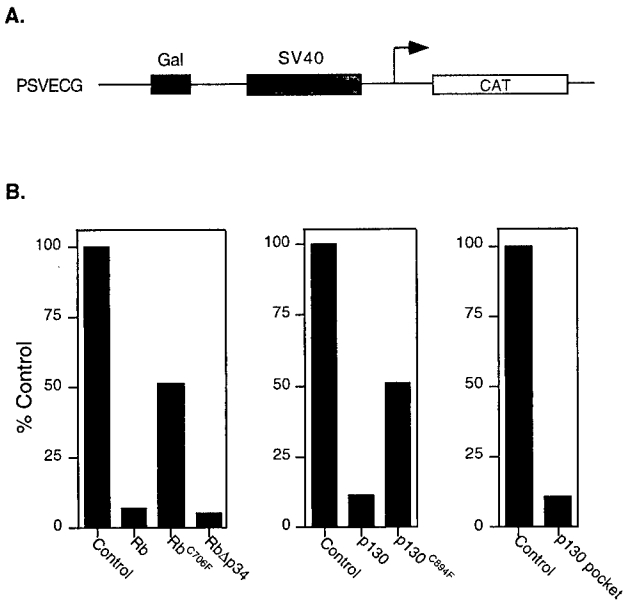

FIG. 1. Rb/p130-mediated repression independent of histone deacetylase recruitment. (*A*) Schematic representation of the pSVECG reporter. Constitutive CAT expression is driven by the SV40 promoter/enhancer. Upstream Gal4 sites provide a binding site for Gal4 DNA biding domain fusion proteins. (*B*) C33A cells were transiently transfected with 1 μg of β-gal, 0.5 μg of the pSVECG reporter, and 2 μg of Gal4-Rb, Gal4-RbΔp34, Gal4-p130, or Gal4-$p130^{C894F}$ or 5 μg of Gal4-Rb^{C706F}. Western blotting was performed to verify that transfected constructs expressed equal protein. For the repression assay using the pocket domain, 0.5 μg of pSVECG was transfected with 3 μg of Gal4-p130 pocket. As controls, C33A cells were transfected with 1 μg of β-gal, 0.5 μg of pSVECG reporter, and 2 or 3 μg of a vector encoding a Gal4 DNA binding domain (control vector). Cells were harvested 40 hours posttransfection, and CAT activity was assayed. β-gal values were used to normalize for transfection efficiency. Results of typical experiments are shown.

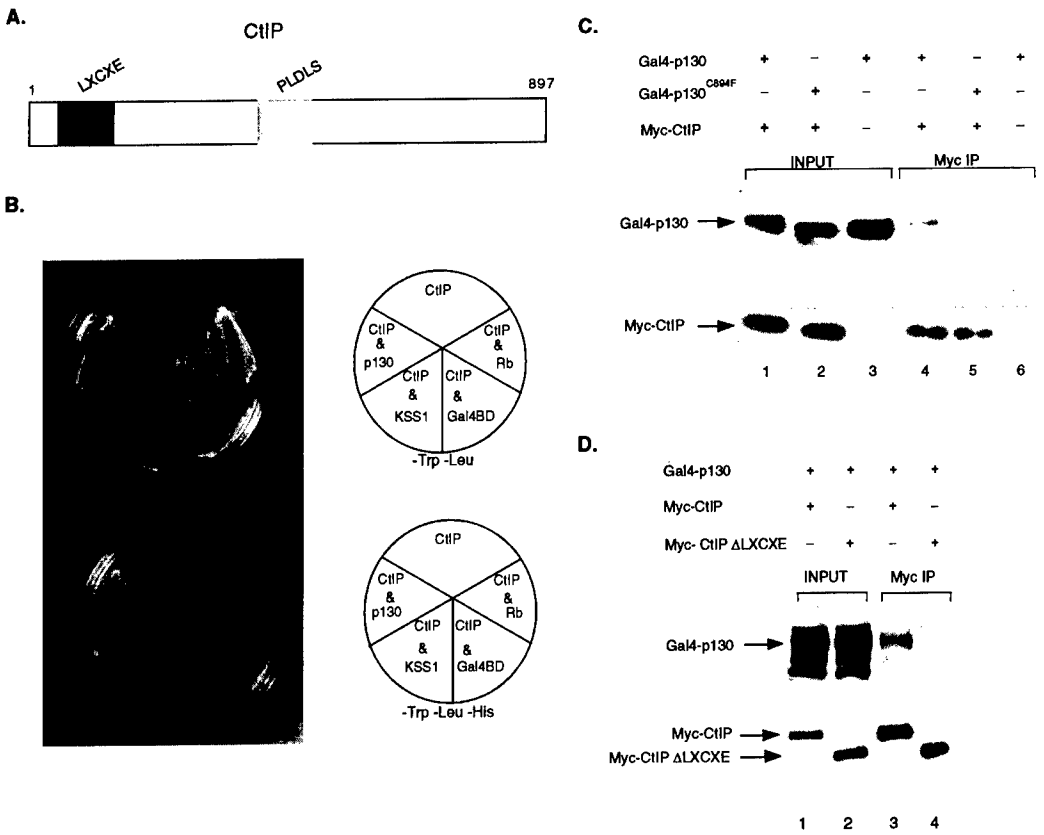

FIG. 2. Rb/p130 Interaction with CtIP. (*A*) Schematic representation of the CtIP protein. The position of an LXCXE sequence motif and a PLDLS sequence motif within the 897-aa CtIP protein are indicated. (*B*) HF7C yeast were transformed with plasmid encoding the Gal4AD-CtIP fusion protein alone or with a Gal4BD-p130, Gal4BD-Rb, Gal4BD-KSS1, or the empty Gal4BD vector. Yeast were streaked on nonselective media lacking Trp and Leu and on media that lacks Trp, Leu, and His that is selective for protein/protein interactions. (*C*) C33A cells were transfected with 10 μg of Myc-CtIP and 10 μg of either Gal4-p130 or Gal4-p130^{C894F}. Cells were harvested 40 hours posttransfection and were lysed in IP buffer. Ten percent of the extract was loaded in input lanes 1–3. Myc antibody (Santa Cruz Biotechnology, 9E10) was used to immunoprecipitate Myc-CtIP. p130 antibody (Santa Cruz Biotechnology) was used in Western blotting to detect p130 in the immunoprecipitates (lanes 4–6). The blot was stripped and reprobed with Myc antibody to verify that equal amounts of Myc-CtIP were immunoprecipitated (lanes 4–6). (*D*) C33A cells were transfected as in *C* with 10 μg of Gal4-p130 and 10 μg of either Myc-CtIP or Myc-CtIP ΔLXCXE. Ten percent of the extract was loaded in input lanes 1 and 2. Myc antibody was used to immunoprecipitate Myc-CtIP. p130 antibody was used in Western blotting to detect p130 in the immunoprecipitates (lanes 3 and 4). The blot was stripped and reprobed with Myc antibody to verify that equal amounts of Myc-CtIP were immunoprecipitated (lanes 3 and 4).

sequence reveals a PLDLS motif within the C-terminal region of the protein (see Fig. 2*A*). As such, we have generated a deletion mutant lacking this sequence and have tested the ability of the mutant to interact with CtBP. As shown in Fig. 4*A*, deletion of the PLDLS sequence in CtIP abolished the interaction with CtBP. We thus conclude that CtIP and CtBP do indeed interact and, like the interaction of the *Drosophila* proteins, the interaction depends on the PLDLS domain of CtIP.

Finally, to explore the role of CtBP in E2F/p130/CtIP-mediated repression, we assayed the effect of the CtIP PLDLS mutation on CtIP-mediated repression. As shown in Fig. 4*B*, the repressing activity of CtIP was clearly impaired by the PLDLS mutation, coincident with the role of the PLDLS sequence in mediating the CtBP interaction. Given the indication that CtIP has the ability to repress transcription when recruited to a promoter, together with the evidence that CtIP can interact with CtBP, we assayed the ability of CtBP alone to function as a transcriptional repressor. A Gal4 DNA binding domain-CtBP fusion was assayed for its ability to repress the SV40 promoter. As shown in Fig. 4*C*, the Gal4-CtBP fusion was equally effective as the Gal4-CtIP fusion protein in repressing the SV40 promoter. Based on these results, we conclude that the CtBP protein does possess transcriptional repressing activity and that the recruitment of CtBP via an interaction with CtIP represents an alternate mechanism for E2F/Rb-mediated repression of transcription.

DISCUSSION

The role of histone deacetylase recruitment in transcription repression, including E2F/Rb-mediated transcription repression, has now been shown in multiple instances (13–15, 27). Nevertheless, it is also clear that other mechanisms, functioning independently of HDAC recruitment, must play a role in repression. The data we present here now describe at least one additional mechanism for E2F/Rb-mediated repression that involves the recruitment of the CtIP/CtBP corepressor complex.

Alternate Mechanisms of E2F/Rb-Mediated Repression. The CtBP protein has been implicated in several forms of transcription repression including the recent studies of the *Drosophila* proteins Hairy, Knirps, and Snail (24–26). In each case, CtBP is recruited to a promoter through the interaction with a PLDLS-containing protein. The results we now present here demonstrate that the mammalian CtBP protein can be recruited to a target promoter through an interaction with CtIP, which in turn interacts with Rb or p130. Although the majority of the PLDLS-containing proteins that are known to interact with CtBP are DNA binding proteins, including Hairy, Knirps, and Snail, there is no evidence to suggest that CtIP has intrinsic DNA binding activity. Rather, CtIP appears to act as a bridging protein, bringing the CtBP corepressor to a promoter through the interaction with Rb or p130 and then E2F (Fig. 5). The observation that CtIP contains distinct motifs that

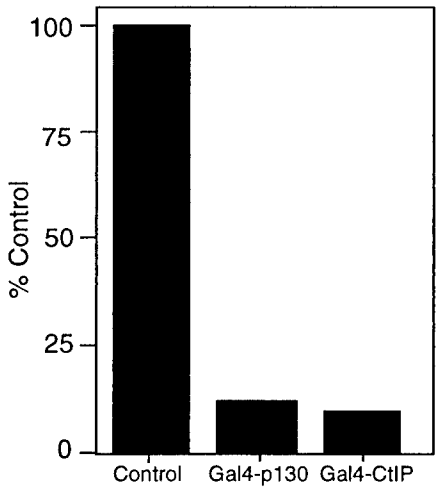

FIG. 3. CtIP is a transcriptional repressor. C33A cells were transiently transfected with 1 μg of β-gal, 0.5 μg of the pSVECG reporter, and 2 μg of Gal4-p130 or 20 μg of Gal4-CtIP. Western blotting was performed to verify that transfected constructs expressed equal protein. As controls, C33A cells were transfected with 1 μg of β-gal, 0.5 μg of pSVECG reporter, and 2 μg of a vector encoding a Gal4 DNA binding domain (control vector). Cells were harvested 40 hours posttransfection, and CAT activity was assayed. β-gal values were used to normalize for transfection efficiency. Results of a typical experiment are shown.

can mediate Rb/p130 binding (LXCXE) as well as CtBP binding (PLDLS) provides a mechanism by which CtIP could serve to bridge the two sets of proteins.

Although CtIP does recruit CtBP, and this can serve as a mechanism for transcriptional repression, it is also possible that other proteins interact with CtIP, possibly leading to other events of transcriptional repression. In this regard, it is of interest to note that, although the recruitment of CtBP coincides with repression by Hairy as well as BKLF, each of these proteins appears to interact with other factors to establish a more complete repression (26, 28). Possibly, the fact that the CtIP PLDLS mutation did not completely abolish repression might suggest that other activities of CtIP could contribute to full repression.

Yet to be determined is the precise mechanism by which CtBP might effect a repression of transcription. Although there has been one report suggesting an interaction of CtBP with histone deacetylase (29), this is unlikely to be the primary mechanism of CtBP-mediated repression given our observations and the observations of others that the SV40 promoter, shown to be repressed by CtBP, is relatively insensitive to histone deacetylase (13). An alternative possibility stems from recent observations that CtBP interacts with the human polycomb proteins (30). Polycomb proteins have been shown in *Drosophila* to be important in repression of certain homeotic genes. Although the mechanisms of this repression are unclear, current models speculate that the PcG proteins can package regions of DNA into heterochromatin-like structures (30).

Multiple Roles for E1A in Affecting Cellular Transcription. Human CtBP was originally identified as a phosphoprotein that associates with the C terminus of E1A (17). The C-terminal E1A sequences that are involved in the CtBP interaction are well conserved among adenovirus serotypes, implying a functional importance. Nevertheless, the analysis of function of these sequences has been somewhat confusing. Some experiments suggest that the C-terminal E1A domain functions to suppress cell transformation in conjunction with an activated Ras protein (23). That is, mutation of the E1A C terminus, which include the domain responsible for binding to CtBP, leads to enhanced oncogenicity in conjunction with Ras.

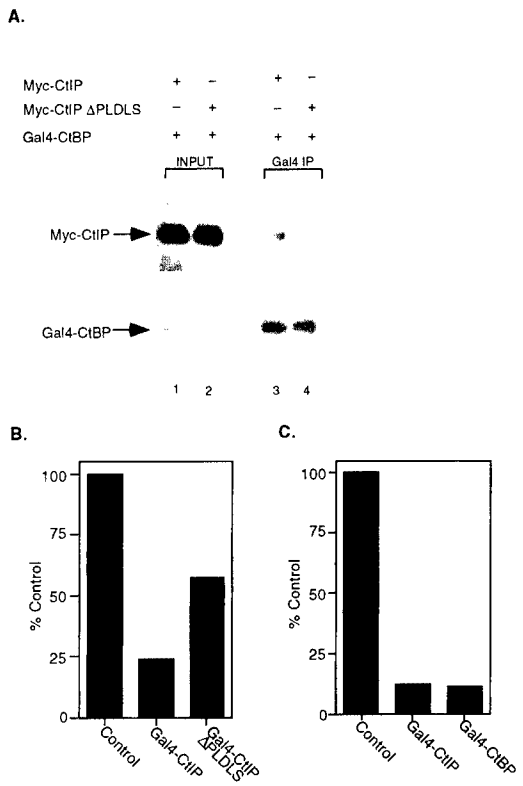

FIG. 4. p130/CtIP-mediated repression involves the recruitment of the CtBP corepressor. (*A*) C33A cells were transfected with 10 μg of Gal4-CtBP and 10 μg of either Myc-CtIP or Myc-CtIP ΔPLDLS. Cells were harvested 40 hours posttransfection and were lysed in IP buffer. Ten percent of the extract was loaded in input lanes 1 and 2. Gal4 DNA binding domain antibody (Santa Cruz Biotechnology, monoclonal) was used to immunoprecipitate Gal4-CtBP. Myc antibody (Santa Cruz Biotechnology, 9E10) was used in Western blotting to detect CtIP in the immunoprecipitates (lanes 3 and 4). The blot was stripped and reprobed with Gal4 DNA binding domain antibody to verify that equal amounts of Gal4-CtBP were immunoprecipitated (lanes 3 and 4). (*B*) C33A cells were transiently transfected with 1 μg of β-gal, 0.5 μg of the pSVECG reporter, and 2 μg of Gal4-CtIP or Gal4-CtIP ΔPLDLS. Western blotting was performed to verify that transfected constructs expressed equal protein. As controls, C33A cells were transfected with 1 μg of β-gal, 0.5 μg of pSVECG reporter, and 2 μg of control vector. Cells were harvested 40 hours postinfection, and CAT activity was assayed. β-gal values were used to normalize for transfection efficiency. Results of typical experiments are shown. (*C*) Same as in *B* except that cells were transfected with 1 μg of β-gal, 0.5 μg of pSVECG reporter, and 2 μg of Gal4-CtBP or 20 μg of Gal4 CtIP.

In contrast, other experiments have provided evidence for a role for these sequences in the immortalizing function of E1A as well as to collaborate with adenovirus E1B in transformation (31). Although the basis for the apparent discrepancy in these results is unclear, the latter findings, indicating a requirement for the C-terminal domain in E1A function in immortalization and transformation with E1B, are certainly consistent with the findings that this domain interacts with CtBP and thus would disrupt the formation of the E2F-p130-CtIP-CtBP repressor complex. It is interesting to note that both E1A and CtIP contain LXCXE and PLDLS motifs, implying that the two proteins target the same factors, Rb and CtBP, and perhaps compete for their binding. In this way, E1A would be seen to disrupt transcriptional repression in three complementary fashions—either the inhibition of Rb family protein interaction with E2F, the inhibition of the interaction of Rb with the CtIP/CtBP complex, or the inhibition of the CtBP repressor with the E2F complex. Interestingly, two recent reports describe yet another mechanism for E1A action that involves a direct inhibition of histone acetyl transferase

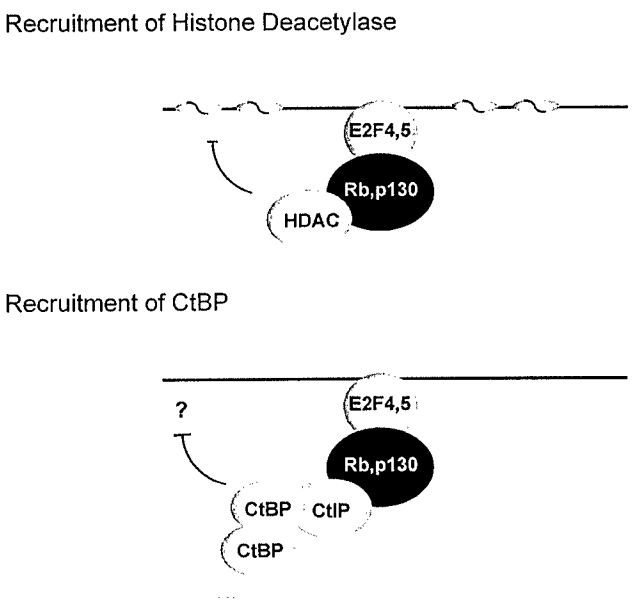

FIG. 5. Alternative mechanisms for Rb/p130-mediated repression. E2F target promoters can be repressed in two fashions. Histone deacetylase is recruited to promoters that contain E2F/Rb or E2F/p130 complexes through an interaction with Rb or p130. Histone deacetylase then modifies the histones proximal to the promoter, causing transcriptional silencing. Rb and p130 recruit CtIP/CtBP to E2F complexes. CtIP bridges the interaction between CtBP and the E2F/Rb complex. CtBP, most likely acting as a dimer, then functions by an undetermined mechanism to mediate repression.

activity (32, 33). In addition to its ability to disrupt complexes involving the p300 protein, these two reports demonstrate that the direct interaction of E1A with either p300/CBP or PCAF leads to an inhibition of histone acetylase activity. As such, it appears that the E1A protein has evolved a series of distinct activities to affect transcription through an alteration of chromatin structure.

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The CtBP Co-Repressor Inhibits Histone Acetylase Activity of CBP

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Summary

E2F transcription activity includes a family of DNA binding activities that function both as transcriptional activating proteins as well as transcription repressors. One group, including the E2F4 and E2F5 proteins, specifically associates with the Rb related p130 protein in quiescent cells, and functions to repress transcription of various genes encoding proteins important for cell growth. A series of recent reports has now provided evidence that Rb-mediated repression involves both histone deacetylase-dependent and independent events. Our previous results suggest that one such mechanism for Rb-mediated repression, independent of recruitment of histone deacetylase, involves the recruitment of the CtBP co-repressor, a protein now recognized to play a widespread role in transcriptional repression. We now show that the CtBP protein inhibits the histone acetylase activity intrinsic to the CBP transcription co-activator, dependent on a domain of CtBP that is required for transcription repression.

Introduction

E2F transcription activity involves a family of DNA binding activities that function both in transcriptional activation and transcriptional repression (1, 2). The E2F1-3 proteins appear to function as transcriptional activators whereas E2F4 and E2F5, in conjunction with Rb family proteins, appear to act as transcriptional repressors. Initial studies of Dean and colleagues demonstrated that E2F/Rb-mediated repression was a dominant event, capable of shutting off an otherwise active promoter (3, 4). A series of reports has provided evidence that one mechanism for this Rb-mediated repression involves the recruitment of histone deacetylase to E2F-site containing promoters, presumably resulting in an alteration of chromatin conformation that hinders transcription (5-7).

Several observations suggest that additional events may contribute to the repression. For example, many genes subject to E2F/Rb-mediated repression are not de-repressed by treatment with the histone deacetylase inhibitor trichostatin A (TSA) (5). Moreover, Rb mutants that can no longer interact with HDAC are still capable of repressing transcription (8). Based on these observations, it would appear that a histone deacetylase independent mechanism of transcriptional repression contributes to the Rb control of transcription.

Employing a yeast two hybrid screen to identify proteins that specifically interact with the Rb-related p130 protein, we identified a protein known as CtIP that interacts with the p130 pocket domain (9). Similarly, others have shown that the CtIP/Rim protein interacts with Rb in vivo (10). CtIP functions as a transcriptional co-repressor in part by recruiting CtBP, an adenovirus E1A-interacting protein (9, 11, 12). Other work has shown that CtBP functions as a co-repressor in Drosophila (13-16) and various experiments have demonstrated a role for CtBP as a transcriptional repressor in mammalian cells (9, 14-22). Nevertheless, despite the clear

evidence for a role of CtBP in transcriptional repression, a mechanistic basis for this repression, whether in the context of E2F/Rb or other transcription regulatory proteins, has not been elucidated. We now describe experiments that demonstrate that CtBP inhibits the histone acetylase activity of the p300/CBP class of transcriptional co-activators, dependent on CtBP sequences that are critical for transcription repression activity. It thus appears that E2F/Rb-mediated repression can involve complementary events that together lead to a loss of histone acetylation at target promoters.

Materials and Methods

Cell culture. C33A cells and 293 cells were grown in DMEM with 10% fetal bovine serum. Sf9 cells were grown in suspension in HyQ SFX-insect without serum.

Plasmids and reagents. The SV40 promoter with upstream Gal4 sites (pSVECG) was a kind gift from D. Dean (4). The MLP and 14D promoters with Gal4 sites were a kind gift from D. Dean and R. Eisenman. The Tk-luc promoter with Gal4 sites was a kind gift from D. Reinberg. The Gal4p130, Myc-CtIP, Gal4BD CtBP, and Gal4AD CtIP constructs were made as described (9). Gal4BD CtBP was made by PCR using 5'-AAAAGAATTCATGGGCAGCTCGCACTT GCT-3' and 5'-AAAATCTAGACTACAACTGGTCACTGGCGT-3' as primers using the CtBP clone (kind gift from G. Chinnadurai) as a template. Gal4BD CtBP$^{\Delta 1\text{-}10}$ was created by PCR using 5'-AAAAGAATTCATGCTGCCGCTTGGCGTCCGACCT-3' and 5'-AAA ATCTAGACTACAACTGGTCACTGGCGT-3' as primers. Gal4BD CtBP$^{\Delta 1\text{-}30}$ was created by PCR using 5'-AAAAGAATTCATGCCCCTGGTGGCATTGCTGGA-3' and 5'AAAATCT AGACTACAACTGGTCACTGGCGT-3' as primers. Gal4BD CtBP$^{\Delta 1\text{-}40}$ was made by PCR using 5'-AAAAGAATTCATGCCCATCCTGAAGGACGT-3' and 5'-AAAATCTAGACT ACAACTGGTCACTGGCGT-3' as primers. Gal4BD CtBP$^{\Delta 1\text{-}70}$ was made by PCR using 5'-AAAAGAATTCATGGCTGTGGGGGCCCTGATGGTA-3' and 5'-AAAATCTAGACT ACAACTGGTCACTGGCGT-3' as primers. Gal4BD CtBP$^{\Delta 1\text{-}120}$ was made by PCR using 5'-AAAAGAATTCATGGCGTCTGTGGAGGAGACGGCC-3' and 5'-AAAATCTAGACT ACAACTGGTCACTGGCGT-3' as primers. All PCR products were cloned using EcoRI-Xba into the same sites in pcDNA3 or pcDNA3-Gal4BD.

The CtBP baculoviruses were created by cloning CtBP or the CtBP mutants into the Eco RI – Xba I sites of the pFASTBAC HTa vector. The CtBP and CtBP mutant yeast expressing plasmids were created by cutting CtBP out of the pFASTBAC HTa vector with EcoRI-XhoI and cloning into pGAD424 or pGBT9. Virus was made according to the BAC-TO-BAC Baculovirus Expression Systems protocol (Gibco Life Technologies). The Flag-CBP virus as well as E1A and GST-CBP were made as described (23),(24, 25).

Repression assays. C33A cells were transiently transfected by the calcium phosphate method with the described DNA plasmids. A β-gal expressing plasmid (1 μg) was cotransfected as a control for transfection efficiency. After 15 hr, cells were washed 2X with PBS and allowed to recover in DMEM with 10% serum. 40 hours post transfection cells were harvested and CAT assays were performed as described in Maniatis with the following modifications: Extracts were not heat inactivated. CAT assay reaction mixture includes 75 μl extract, 75 μl 1M Tris.Cl (7.8) 1 μl ^{14}C-labled chloramphenicol (1 mCi/ml), and 30 μl of acetyl coenzyme A (3.5 mg/ml in H_2O). Reactions were incubated at 37^{o} for 3.5 hours. Raw CAT values were normalized relative to the vector alone control β-gal values. β-gal was measured by adding 10 μl of the extract prepared for the CAT assays to 590 μl of 0.1mg/ml CPRG in Z buffer. Absorbance was measured at 570. In all instances of comparison, westerns were performed to determine that equal protein levels are expressed.

Yeast two-hybrid assays. The yeast two hybrid is performed as recommended in the CLONTECH protocol.

Baculovirus expressed proteins. Purified baculovirus expressed proteins were made by infecting 50 mls of 2 x 10^6 cells/ml SF9 cells with a given virus. 48 hours post infection, cells were harvested and lysed in 50mM Tris-HCl, 1% NP40, 10mM BME, 1mM PMSF, 2 μg/ml Pepstatin, and 2 μg/ml Aprotinin. Extracts were then incubated for 30 minutes at 4 degrees with 1ml of a 50% slurry of Nickel beads. Beads were loaded onto a column and washed with lysis buffer. Protein was eluted in 20mM Tris-HCL, 100mM KCl, 100mM imidizole, 10% glycerol, and 10mM BME. 0.5 ml fractions were collected and peak fractions 2,3 and 4 were pooled.

In vitro protein interaction assay. CtBP was synthesized by the T7 TNT Quick Coupled Transcription/Translation System (Promega). BL21 cells expressing GST or GST-CBP (amino acids 720-1677) were lysed in 150 mM NaCl, 50 mM Tris, 0.5% NP40, pepstatin, leupeptin, AEBSF, and aprotinin. Cells were sonicated for 20 sec and then centrifuged at 4°C. Equal amounts of GST or GST-CBP were incubated with glutathione sepharose beads for 1 hr and then the beads were washed 3 times in lysis buffer. An aliquot (10%) of the in vitro translation mix of CtBP was removed and the remainder was diluted to 600 μl with lysis buffer. The diluted translation mix was split in half and incubated with GST or GST-CBP for 6 hr at 4°C. Beads were washed 3 times in lysis buffer, and then analyzed by SDS gel electrophoresis. Gels were fixed, enhanced, dried, and then exposed to film for 2 hr.

Co-immunoprecipitation assays. Sf9 cells were infected with baculovirus expressing Flag-tagged CBP or Gal4DNA binding domain-tagged CtBP. Cells were lysed in 500 µl of lysis buffer (150 mM NaCl, 1% NP40, 50 mM Tris, PMSF, Leupeptin, Pepstatin, Aprotinin). Extracts were precleared with protein A+G beads for 1 hr at 4° C. Extracts were then incubated for 1.5 hr at 4° C with Protein A+G beads that were prebound to 1 µg of anti Gal4DBD antibody (Santa Cruz). Beads were then washed 3 times in 1 ml of lysis buffer and analyzed in an SDS acrylamide gel. Western analysis was performed using polyclonal CBP antibody (Santa Cruz).

Histone acetylase assays. Approximately 150 ng of baculovirus purified Flag-CBP was preincubated with 2.5 to 15 µg of BSA, baculovirus purified CtBP, or CtBP mutants in a 15 µl volume with buffer containing 50mM Tris-HCl, 10% glycerol, 1mM DTT, 1mM PMSF, 0.1mM EDTA, 75mM NaCl, and 10mM Butyric Acid for 20 minutes at 30 degrees. After preincubation, 25 µg histones and 125 µCi of ^{3}H-acetyl CoA were added and the volume raised to 30 µl keeping the buffering conditions constant. The reaction was allowed to proceed at 30° C for 20 more minutes after which the reactions were immediately boiled and run on a 15% acrylamide gel. The gel was fixed, amplified, dried and exposed to film. After exposure, the gel was rehydrated and stained with Coomassie blue.

Results

Recent work has shown that the CtBP protein, either from mammals or Drosophila, plays a role as a transcription co-repressor through physical interactions with a variety of promoter specific transcription factors. CtBP was originally identified as a protein that bound to the C terminus of E1A (C terminal binding protein) (12). E1A also interacts with the Rb family proteins and in so doing, disrupts the growth suppressing activity of the Rb proteins (26). As such, we speculated that E1A might also disrupt the transcription repressor function of CtBP. We have investigated this possibility using a Gal4-CtBP fusion protein that eliminates the contribution of Rb to CtBP repression. Although expression of the Gal4-CtBP fusion protein results in a substantial repression of the major late promoter (MLP) when assayed in C33A cells, a very different result is observed when the same assay is carried out in 293 cells. In this case, expression of Gal4-CtBP did not repress MLP but in fact led to a modest increase in promoter activity (Figure 1A). 293 cells express high levels of E1A, suggesting the possibility that E1A might disrupt the ability of CtBP to mediate repression. To directly test this possibility, we again performed the Gal4-CtBP repression assay in C33A cells, this time adding varying amounts of E1A. While the addition of E1A together with Gal4-CtBP did not induce CtBP mediated activation of transcription, significant derepression of the MLP was observed (Figure 1B). It would thus appear that E1A does indeed disrupt the ability of CtBP to repress transcription. Although the CtBP-mediated increase in transcription in 293 cells could be a consequence of E1A interaction with CtBP, converting it from a repressor to an activator by supplying an activation function, it is also possible that CtBP might activate transcription in a cell dependent fashion as suggested by other recent studies (27).

Previous work has shown that the interaction of E1A with CtBP is dependent on a PLDLS sequence in the C terminal region of E1A (12, 28). In light of the finding that E1A can disrupt CtBP-mediated repression, it seemed possible that CtBP might require the domain through which it interacts

with PLDLS motif containing proteins in order to mediate repression. Previous work has identified a centrally located dimerization domain within the 439 amino acid CtBP protein as well as a large and poorly defined domain necessary for interaction with PLDLS proteins that includes most of the N-terminus of the protein (21). Given the possible role of the PLDLS-interaction in mediating repression, we generated a series of N terminal deletions to more precisely map the domain involved in PLDLS interaction and to identify the CtBP sequences important for transcriptional repression. Five such mutants were created lacking the first 10, 30, 40, 70 or 120 amino acids of the CtBP protein (Figure 2A). These mutants were first tested in a two-hybrid assay for their ability to interact with a PLDLS motif-containing protein, in this case the CtIP protein. While wild type CtBP and mutants lacking the first 10 or 30 amino acids retained the ability to interact with CtIP, mutants lacking the first 40, 70, or 120 amino acids all failed to interact with CtIP (Figure 2B). As a control, we find that all of the mutants can dimerize with wild type CtBP and are therefore functionally expressed in yeast.

We next assayed the ability of these CtBP mutants to repress transcription in C33A cells. Wild type and mutants of CtBP were expressed as Gal4 DNA binding domain fusions along with the SV40 promoter containing upstream Gal4DNA binding domain sites as a reporter. While the SV40 promoter was dramatically repressed by the wild type and mutants with the first 10 or 30 amino acids deleted, mutants containing deletions of the first 40, 70 or 120 amino acids were significantly impaired in their ability to repress the SV40 promoter (Figure 2C). Similar repression results were obtained using a variety of other active promoters including the major late promoter, the 14D promoter, the herpesvirus thymidine kinase promoter, and the basal promoter pG5 Luc (Figure 2E). Western analysis reveals that all fusion proteins were expressed at relatively equal amounts (Figure 3D). These results thus define an N terminal domain that is essential for CtBP mediated transcription repression that also coincides with a PLDLS-interacting domain (PID).

Due to the importance of the PID domain in mediating repression by CtBP, we examined the possibility that CtBP might repress transcription by targeting a cellular factor that contains a PLDLS-like motif. By examining the group of proteins that are currently known to interact with CtBP through a PLDLS-like motif, it appears that most CtBP interacting proteins contain P-X-[D/S/N]-L-[S/T/V] motif (20). We therefore searched the Swissprot + SPT.EMBL, PIR, and ProClass protein databases (29, 30) for proteins that contain this motif. The search revealed a large number of proteins from all species, containing this motif. We limited the number of candidate proteins by examining only human proteins with established function in transcription regulation (Table 1). Among this list were many transcription factors known to act as negative regulators of transcription, several of which are already known to bind CtBP. Interestingly, we also identified several transcription co-repressors, such as the proteins N-COR, TGIF and methyl CpG binding protein, proteins found in complexes with histone deacetylase, and Tif1α and Rip140, nuclear receptor interacting repressors. As CtBP has been described to interact with HDAC and to exhibit sensitivity to TSA on certain promoters (19, 22, 31, 32), it is possible that one mechanism of CtBP mediated transcription repression is through a HDAC dependent mechanism potentially through interactions with one or more of these proteins.

Several co-activators were also found to contain P-X-[D/S/N]-L-[S/T/V] motifs such as the 60 kd subunit of the mammalian Swi/Snf complex, a chromatin remodeling ATPase involved in transcription activation and the thyroid hormone receptor co-activator. An interesting set of co-activating PLDLS-motif containing proteins are the histone acetyltransferases, p300, CBP, GCN5, and TAFII-250. p300 and CBP contain a PMDLS motif like that found in the *Drosophila* Knirps protein. The PMDLS motif is located in the bromodomain, just upstream from the histone acetylase catalytic domain (Fig. 3A). Interestingly, this motif is conserved in several bromodomain containing proteins. The finding that CBP and p300 may contain a CtBP interacting motif was intriguing given the recent data that the E1A, E6, and

Twist proteins can inactivate the acetyltransferase activity of p300/CBP (33-35). To directly test for an interaction, CtBP and CBP were co-expressed by baculovirus infection of Sf9 cells and then assayed for interaction by co-immunoprecipitation assays of cell extracts. The CBP western shown in Figure 3B reveals that Flag-CBP could be immunoprecipitated with the Gal4DBD antibody only when co-expressed with the Gal4DBD CtBP protein indicating that CtBP and CBP can indeed interact when co-expressed in the Sf9 cells. As a further test for interaction, ^{35}S-labeled CtBP and the PLDLS binding deficient mutant CtBP$^{\Delta 1\text{-}120}$ were produced by in vitro transcription/translation and then assayed for interaction with a bacterially expressed GST-CBP (aa 720-1677) fusion protein. Protein bound to either the GST-CBP fusion or GST alone was analyzed by SDS gel electrophoresis. As shown in Figure 3C, wild-type CtBP did indeed interact specifically with GST-CBP while the CtBP$^{\Delta 1\text{-}120}$ mutant interacted significantly less well.

Given this interaction of CtBP with CBP, together with the role of the intrinsic histone acetylase activity in the function of CBP as a co-activator for many if not all promoters, we measured the effect of CtBP on the histone acetylase activity of CBP. As shown in Figure 4A, pre-incubation of bacculovirus-produced CBP with bacculovirus-produced CtBP followed by addition of histone, and ^{3}H acetyl CoA resulted in a substantial inhibition of acetylase activity. We further examined the dose effect of the CtBP inhibition of CBP. Figure 4B shows that a 20 minute preincubation of CBP with doses of CtBP ranging from 2.5 μg to 15 μg produced an increasingly dramatic inactivation of CBP relative to the BSA (SIGMA and NEB) control. These data indicate that the CtBP corepressor can significantly inactivate the histone acetylase activity of CBP in vitro.

To determine whether the inactivation of CBP histone acetylase activity reflects the CtBP-mediated transcription repression, we assayed the baculovirus-produced CtBP proteins for their ability to affect acetylase activity. We preincubated BSA, CtBP, CtBP$^{\Delta 1\text{-}10}$ or CtBP$^{\Delta 1\text{-}120}$ with bacculovirus-

produced CBP for 20 minutes followed by the addition of histone and ^{3}H-acetyl CoA. While CtBP and CtBP$^{\Delta 1\text{-}10}$ dramatically inhibited the acetylation of histones by CBP relative to the BSA control, CtBP$^{\Delta 1\text{-}120}$ was severely impaired in its ability to inactivate CBP (Figure 4C). The same result was obtained in multiple experiments and with different preparations of CtBP and the mutant proteins. As an additional control, the same experiment was carried out using boiled samples of BSA, CtBP and the CtBP mutants. Figure 4D shows that boiled CtBP is incapable of inactivating CBP thereby implicating the CtBP protein in the inactivation of HAT activity. These results correlate the ability of CtBP to repress transcription with its ability to inactivate CBP, indicating that one mechanism of CtBP mediated transcription repression is through the inactivation of acetylase activity of CBP.

Discussion

The role of localized histone acetylation, as a mechanism for control of transcription activity has now been shown in multiple instances. This includes both the recruitment of histone acetylases as a mechanism for transcription activation, as well as the recruitment of histone deacetylases as a mechanism of transcriptional repression. Our finding that the CtBP co-repressor can physically interact with and inhibit the HAT activity of CBP provides yet another mechanism for transcription control through acetylation. Since CBP-like co-activators with intrinsic HAT activity are likely to be a common component of many transcriptional activation events, the role of a histone acetylase inhibitor such as CtBP could be of wide-spread importance.

In some instances, CtBP is recruited to a promoter complex through a direct interaction with DNA binding transcription factors. This includes the Drosophila proteins *Snail, Knirps,* and *Hairy* as well as the mammalian proteins such as BKLF, delta EF, Zeb, Net, AREB6, Evi-1, Ikaros, Fog, and HPC2 (13, 15, 17, 21). In other instances, CtBP is recruited to a promoter through an interaction with the CtIP protein as is the case for BRCA1 and Rb (9, 36). Interestingly, CtBP has been described to interact with several short range repressors. These repressors are thought to act over distances of less than 100 bp on upstream activators or the core transcription complex (14). Perhaps then CtBP may function to inactivate CBP or other HATS recruited by other nearby transcription factors or by the basal machinery.

In the case of E2F/Rb-mediated repression, the inhibition of acetylase activity of p300/CBP suggests a complementary event for E2F/Rb-mediated repression. In one case, the recruitment of histone deacetylase can lead to transcriptional repression through the localized alteration of chromatin structure (5-7). This action alone, however, might be expected to be less than complete given the expected continued histone acetylation mediated by promoter-bound

acetylase. Our observation that E2F/Rb can also recruit a histone acetylase inhibitor would then allow a complete inhibition of histone acetylation. Taken together, these data are consistent with the model diagrammed in Figure 5. We propose that E2F/Rb or E2F/p130 complexes can recruit histone deacetylase and/or CtBP to modify chromatin structure at target promoters, blocking the accessibility of the transcription machinery, and thus resulting in repression. Consistent with this model, a mutant Rb that can bind to CtIP but not to HDAC, retains the ability to repress transcription in a TSA insensitive manner (8). The interaction between CtBP and CBP in our assays is weak but specific. Perhaps this binding represents a transient interaction that is stabilized by the presence of other proteins on a promoter scaffold. Alternatively, the interaction of CtBP with CBP may indeed be an intrinsically weak interaction but is promoted through the recruitment of CtBP by DNA binding transcription factors to the vicinity of CBP on a promoter.

It remains to be determined how CtBP might inactivate the HAT activity of CBP. As the CtBP protein is highly related to the D-hydroxy-acid dehydrogenases, one speculative model is that CtBP might bind to CBP and enzymatically alter it. However, mutation of the putative dehydrogenases domain of CtBP does not affect the ability of CtBP to repress transcription nor does it affect the ability of CtBP to inactivate CBP (data not shown; (27)). Alternatively, the similarity of CtBP to dehydrogenase may simply represent the importance of CtBP in dimerization which allows a second PID domain to interact with important co-repressors. Interestingly, recent work has mapped a CtBP repression domain to the C-terminus (27) suggesting the possibility of multiple domains within CtBP that can mediate repression. Possibly, this may represent a cell-type specific repression in which a different domain is important for repression in different cell types. Indeed, cell type is critically important in CtBP function as CtBP has been shown to act as a transcriptional activator in 293 and B78 cell lines (27). Our

finding of an importance of the PID domain in CtBP-mediated transcripition repression indicates that CtBP may simply bind to the bromodomain of a proximal p300/CBP protein and mediate a conformational change affecting the adjacent HAT catalytic domain as is proposed for the E1A protein. As CtBP has also been shown to interact with HDAC, the relative contribution of each of these mechanisms to CtBP mediated repression of target genes remains to be clarified.

Regardless of the mechanism, the complementary nature of this model is intriguing given that most promoters of E2F target genes contain more than one E2F site which often exists close to the core transcription complex binding site. Perhaps the two sites work together to mediate repression, one recruiting HDAC and another recruiting the HAT inactivating protein, CtBP. Alternatively, since Rb has been shown to have the capacity to bind simultaneously to HDAC and BRG1 (37), both of which contain LXCXE motifs, perhaps HDAC and CtBP can co-exist in one E2F-Rb complex. In this manner, the presence of both HDAC and a HAT inactivating protein on the same promoter would insure local chromatin structure containing hypoacetylated nucleosomes and thus transcription repression.

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Table 1. Identification of PXDLS-containing proteins

Protein	Sequence
DNA-binding transcription factors	
Two-handed zinc finger protein ZEB	PLDLS
Transcription factor 8 (Delta EF1)	PLDLS
Negative-regulator of IL-2	PLDLS
cAMP response element binding protein CRE-BP1 (ATF2)	PLDLS
Zinc finger protein FOG-2	PIDLS
DNA-binding protein IKAROS	PEDLS
Ras-responsive element binding protein 1 RREB-1	PIDLS
Elk3	PLNLS
Evi1	PLDLS/PFDLT
AREB6	PEDLT/PLNLS/PLDLS
NF-AT	PLSLT
NFκB	PLDLT
TCF-4	PLSLV
Retinoblastoma protein interacting zinc finger protein RIZ	PLDLS
Transcription co-repressors	
CtBP interacting protein CtIP	PLDLS
5'-TG-3' interacting factor TGIF	PLDLS
Methyl-CpG binding protein 2 MECP-2	PQDLS
Receptor interacting protein 140	PIDLS
Transcription intermediary factor 1-alpha	PMDLS
N-CoR	PENLV
Transcription co-activators	
E1A-associated protein p300	PMDLS
CREB-binding protein CBP	PMDLS
Thyroid hormone receptor co-activating protein	PMDLS
SWI/SNF complex 60 KD subunit	PGDLS
GCN5	PENLT
Basal transcription machinery	
TFIIF	PQSLS
TAFII-250	PQDLT

The SWISSPROT + SPTrEMBL, PIR and ProClass protein databases were searched for instances of the pattern P-X-[D/S/N]-L-[S/T/V] using the IBCP and PIR web sites. This list was then limited to human proteins known to play a role in transcription. These proteins were then grouped in 4 general categories: DNA binding transcription factors, transcription co-repressors, transcription co-activators, or basal transcription machinery.

Figure 1. E1A disrupts CtBP-mediated transcription repression.

A. C33A or 293 cells were transiently transfected with 1μg of CMV β-gal, 0.5μg MLP reporter, and 2μg or 5μg of Gal4-CtBP. Cells were harvested 40 hours posttransfection, and CAT activity was assayed. β-gal values were used to normalize for transfection efficiency. The experiment was repeated at least three times and the results of a typical experiment are shown.

B. C33A cells were transiently transfected as in panel A except 1 μg or 5 μg of E1A plasmid was added where indicated. Percent control for MLP represents expression from the ML promoter vector expressed with and without E1A and set to 100%. The experiment was repeated at least three times and the results of a typical experiment are shown.

Figure 2. Identification of a domain within CtBP essential for PLDLS interaction and transcription repression

A. A schematic representation of CtBP along with the N-terminal deletion mutants.

B. HF7C yeast were transformed with plasmid encoding Gal4DBD-CtBP or each of the mutants along with Gal4AD-CtIP, Gal4AD-CtBP or Gal4AD. Yeast were then plated on media lacking Trp, Leu, and His that is selective for protein/protein interactions. Yeast that grew on selective media were then assayed for the presence of β-gal. An interaction was scored positive if yeast could both grow on selective media and express β-gal.

C. The Gal4-CtBP wild type and mutants were cloned into mammalian expression vectors and transfected into C33A cells with β-gal, and pSVECG (SV40) reporter plasmid. Cells were harvested 40 hours posttransfection, and CAT activity was assayed. β-gal values were used to normalize for transfection efficiency. Results of a typical experiment are shown.

D. Experiment was performed as in C using MLP, PG5LUC basal promoter, 14D promoter, and the Herpes virus TK promoter.

E. Western blot, using Gal4DNA binding domain antibody (Santa Cruz), of C33A transfected Gal4-CtBP and mutants. β-gal was used as a loading control for transfection efficiency.

Figure 3. CtBP binds to CBP

A. Schematic depiction of CBP including the PMDLS-containing bromodomain, the HAT domain, and other regions known to bind various transcriptional regulatory proteins.

B. Sf9 cells were infected with Flag-CBP, 6His CtBP, or both. Extracts were incubated with Protein A+G beads prebound to Gal4 DBD antibody. Beads were washed and then bound material was eluted and separated in an SDS-acrylamide gel. The input is 1% of the total sample. Input exposure is 10% of the IP exposure using ECL chemiluminescence.

C. In vitro translated CtBP or CtBP$^{\Delta 1\text{-}120}$ was incubated with either GST or GST-CBP (amino acids 720-1677), washed three times, and then the bound material was analyzed by SDS gel electrophoresis. The input material represents 1% of the labeled protein used for binding assays. Binding experiment was performed reproducibly in three separate experiments.

Figure 4. CtBP inhibits histone acetylase activity of CBP

A. Baculovirus-expressed Flag-CBP was pre-incubated for 20 min with 15 μg of baculovirus-expressed 6His-CtBP or an equal amount of BSA as a control. ^{3}H-acetyl coenzyme A and 25 μg histone were then added to the reaction which was allowed to proceed for the indicated time. Products were analyzed in a 15% acrylamide gel.

B. Baculovirus-expressed Flag-CBP was pre-incubated for 20 min with 2.5 μg, 5 μg, 10 μg or 15 μg of baculovirus-expressed 6His-CtBP or BSA. ^{3}H-acetyl coenzyme A and histone were then added to the reaction which was allowed to proceed for 20 min.

C. Baculovirus-expressed Flag-CBP was pre-incubated for 20 min with baculovirus-expressed 6His-CtBP, CtBP$^{\Delta 1\text{-}10}$, CtBP$^{\Delta 1\text{-}120}$ or an equal amount of BSA as a control. ^{3}H-acetyl coenzyme A and histone were then added to the reaction which was allowed to proceed for 20 min. This experiment is a representative of 6 separate assays with two different preparations of purified proteins.

D. Assays were carried out as described in panel C except using BSA, CtBP, CtBP$^{\Delta 1\text{-}10}$, and CtBP$^{\Delta 1\text{-}120}$ that were first boiled prior to incubation with CBP.

Figure 5. Complementary mechanisms for E2F/Rb mediated transcription repression

E2F target promoters can be repressed in two fashions. Histone deacetylase is recruited to promoters that contain E2F/Rb or E2F/p130 complexes through an interaction with Rb or p130. Histone deacetylase then modifies the histones proximal to the promoter causing transcriptional silencing. Rb and p130 recruit CtIP/CtBP to E2F complexes. CtIP bridges the interaction between CtBP and the E2F/Rb complex. CtBP, acting as a dimer, then functions by inhibiting p300/CBP bound to the promoter through basal transcription machinery or other transcription factors.

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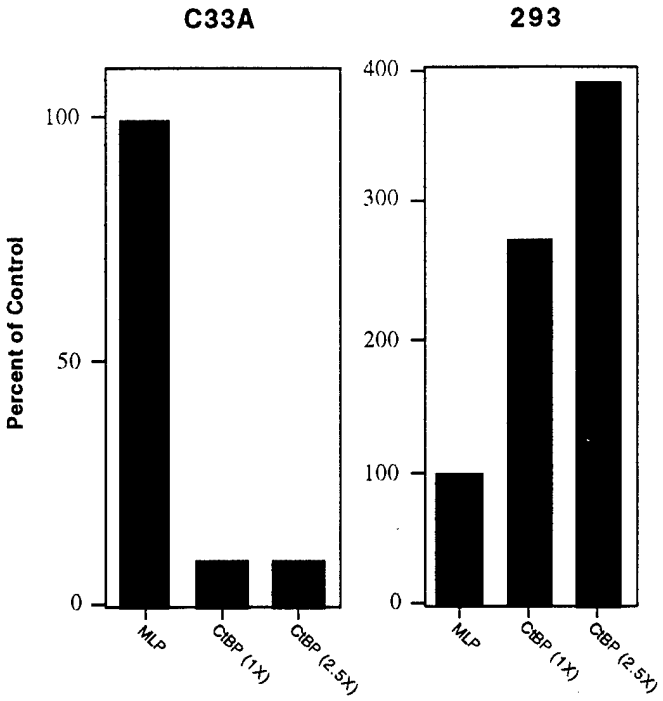
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50
0
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300
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MLP
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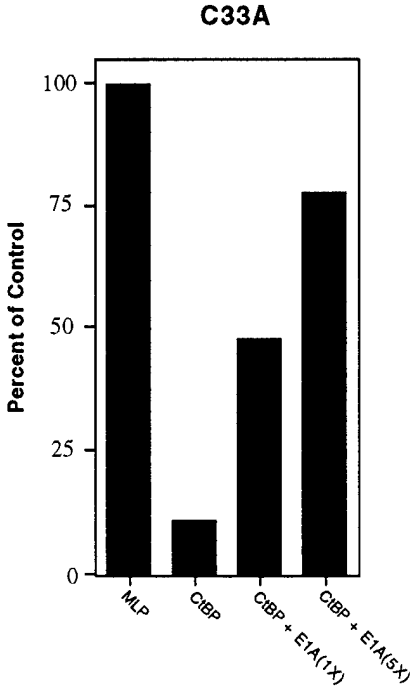
CtBP (2.5X)
MLP
CtBP (1X)
CtBP (2.5X)

B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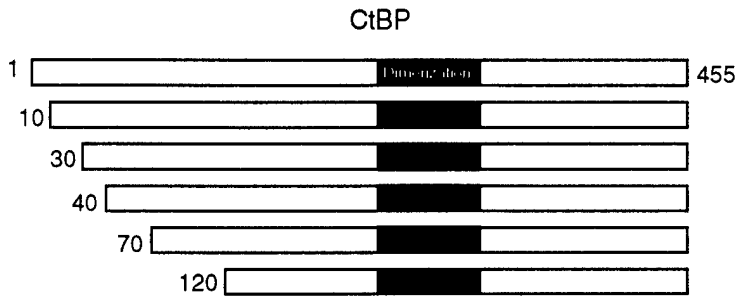
A



B

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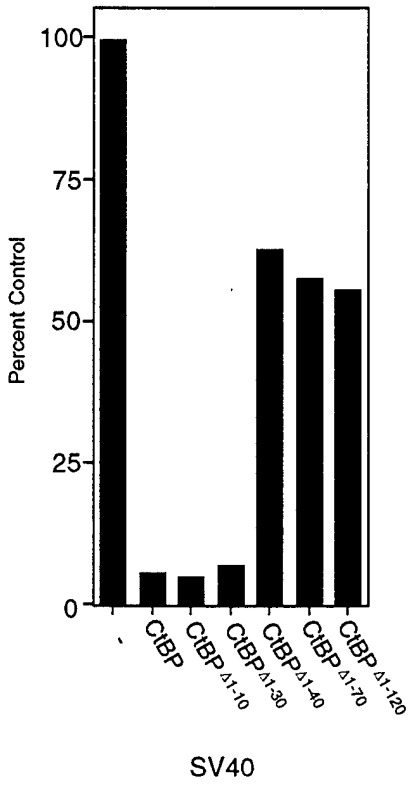
Gal4AD CtBP	+	+	+	+	+	+
Gal4AD	–	–	–	–	–	–

C



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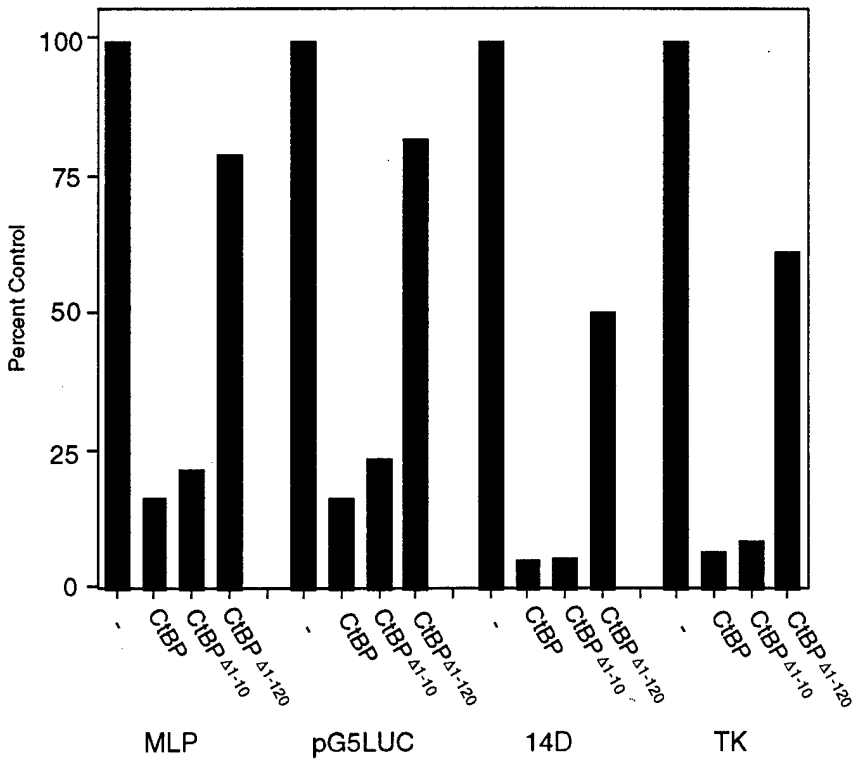

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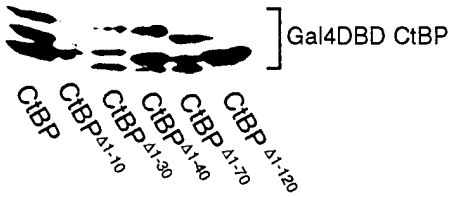

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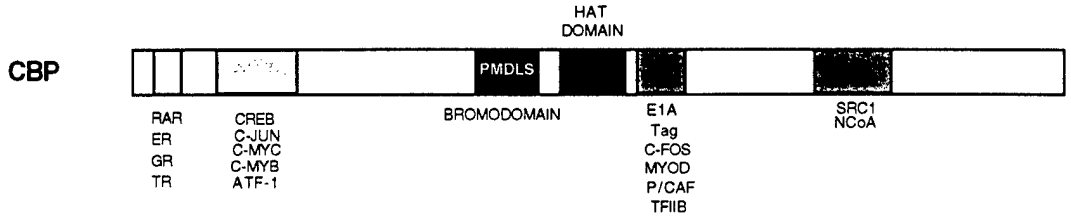
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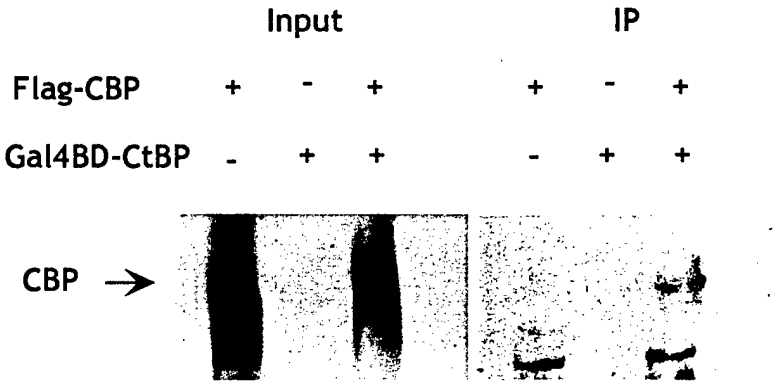
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CBP

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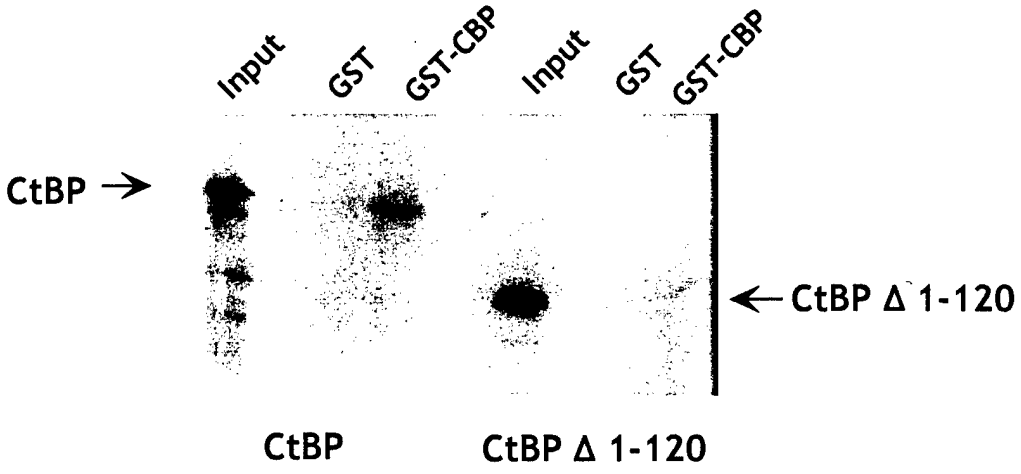
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Input
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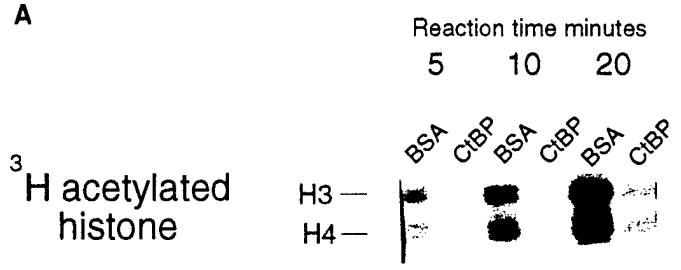
CtBP
CtBP Δ 1-120
CtBP
CtBP Δ 1-120

A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10
20
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CtBP
BSA
CtBP
BSA
CtBP
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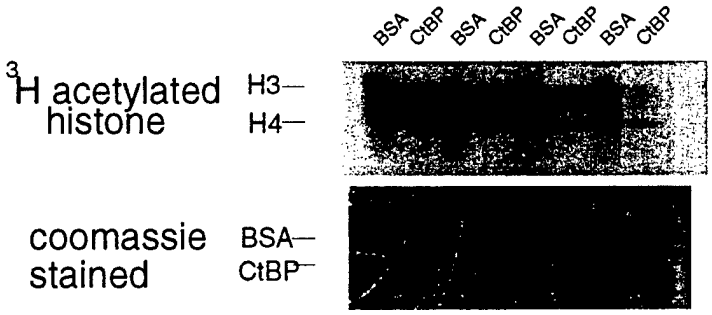
H3
H4

B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CtBP

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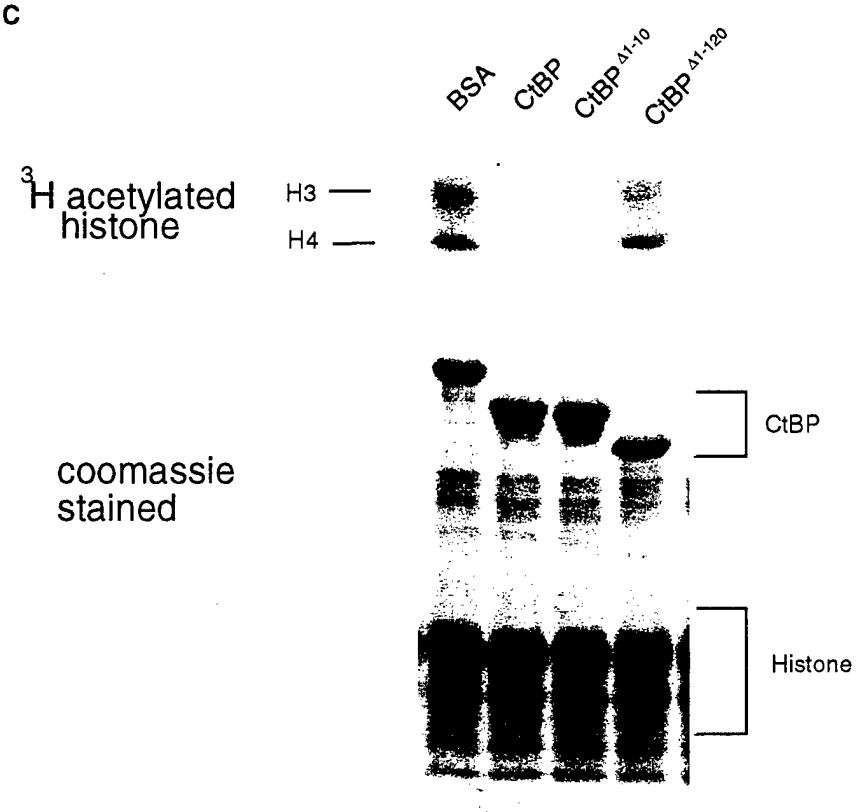
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Histone

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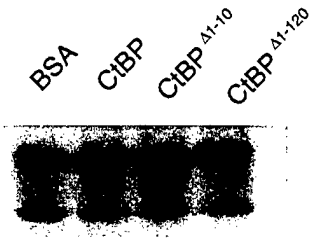
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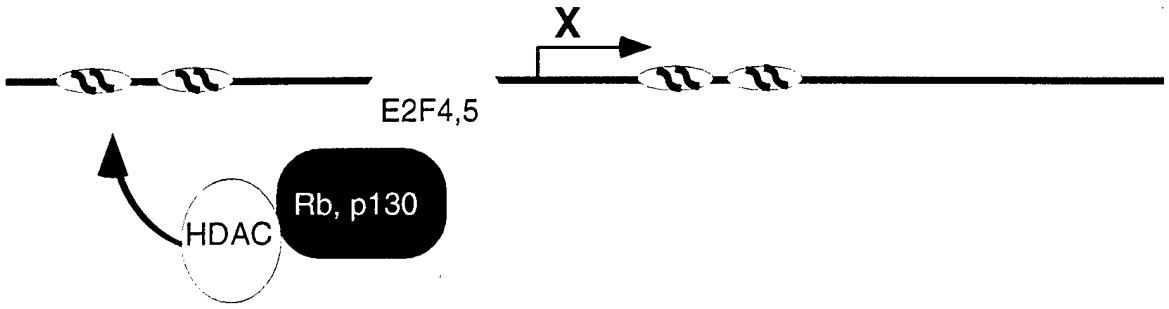
Recruitment of Histone Deacetylase



Recruitment of a Histone Acetylase Inhibitory Complex